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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPLC METHOD FOR DRUG STABILITY STUDIES

Vývoj a validace HPLC metody pro stabilitní studie léčiv

Master's thesis

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Hradec Králové 2012

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Acknowledgements

This Master´s Thesis was carried out in the laboratories of the Department of Pharmaceutical Chemistry at the University of Eastern Finland, Faculty of Pharmacy in Kuopio, Finland, with the support of ERASMUS/SOCRATES exchange program.

"I declare that this thesis is my original author piece. Literature and other sources that were used during the writing process are all listed in References and are properly cited throughout the work. The thesis was not previously used to acquire a Master's or any other degree."

I would like to thank Prof. Seppo Auriola that he gave me an oppurtunity to work on his department in modern laboratories and with modern facilities,. I thank Msc. Marko Lehnoten for guiding and helping me in lab work that I had never done before. For big help with writing process, I thank RNDr. Marie Musilová, and also my family for a support.

Abstract

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Development and validation of stability indicating HPLC method for drug stability studies
Hana Chlubnová

Drugs represent preparations with long shelf-life. From this reason it is necessary to have substances chemically and physically stable and prevent possible degradation or forming complexes during whole shelf-life.
We need to check the stability of preparations. One of the appropriate methods is using of HPLC having several advantages e.g. in simlipicity and availability. Before measuring there is a requirement to determinate proper conditions and validate the chosen method.
In the experimental part of this thesis there are stated conditions and results of 38 measured substances. Based on the results it was chosen one suitable method that was validated for 10 concrete active substances. There are results of stability of these substances after melt-quenching in the last part.

Abstrakt

Univerzita Karlova v Praze
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Katedra farmaceutické technologie

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Léky představují přípravky s dlouhou dobou použitelnosti. Z tohoto důvodu je potřeba, aby látky byly chemicky a fyzikálně stabilní a zabránit tak možnému rozkladu nebo tvorbě komplexů v průběhu celé doby použitelnosti.
Potřebujeme kontrolovat stabilitu přípravků. Jedna z vhodným metod je použití HPLC, které má několik výhod např. jednoduchost a dostupnost. Před měřením je potřeba stanovit vhodné podmínky a validovat vybranou metodu.
V experimentální části této práce jsou uvedeny podmínky a výsledky 38 měřeným látek. Na základě těchto výsledků byla vybrána vhodná metoda, která byla validována pro 10 konkrétních účinných látek. V poslední části jsou uvedeny výsledky stability těchto léčiv po tepelné zátěži.

Abbreviations

ACEi.....angiotension-converting-enzyme inhibitor

ACN.....acetonitrile

ATB.....antibiotics

CD.....cyclodextrin

CI.....confidence interval

CMC.....critical micelle concentration

DSC.....diferencial scanning calorimetry

e.g.....for example

HPLC.....high pressure liquid chromatography

M_r.....molecular weight

NSAIDs.....non-steroidal antiinflammatory drugs

pH.....a measure of the acidity or basicity of an aqueous solution

pKa.....a logaritmic measure of acid dissociation constant

RSD.....relative standard deviation

Rt.....retention time

SD.....standard deviation

$t_{1/2}$, t_{50}.....half life

t_{95}.....shelf life with 5% loss

T_g.....glass transition temperature

T_m.....melting temperature/point

Contents

Goal of the thesis

The goal of the theoretical part is to describe amorphous solids, their properties and preparation in short way then characterize chemical and physical degradation and show possible techniques to improve stability.

The subject of experimental part was to determinate possible degradation of several samples that were modified by melt-quenching. For this purpose we developed and validated an HPLC method that gave us valid results of stability measured compounds.

1. Theoretical part

Active substances used in pharmacetical industry and more other areas are mostly in solid state. They can exist in many forms such as amorphous solids, crystals, polymorphs, hydrates, solvates. They have different chemical, physical, thermal and mechanical properties that lead to a turn in other characteristics (melting point, bioavailibility, stability). Transitions between the forms result in possible clinical and toxicological changes. Scientists have to develop various methods to prevent these changes and find new drug delivery systems to guarantee safety, effectiveness and quality during whole shelf-life.

1. 1 Amorphous Solids

1. 1. 1 Solid states

Crystalline state (1)

It is a solid with a short and a long-range order with atoms or molecules in a fixed lattice arrangement. The main difference between crystalline and amorphous state is in their long-range order. Due to its arrangements it is accessible to use X-ray diffraction analysis. Crystal morphology (2) is general appearance of the faces that show and give the crystals their characteric shape. The habit is the general shape as given by relative length of the major axes. These two characteristics depend on surrounding conditions. Crystals exist in seven systems (cubic, tetragonal, orthorhombic, monoclobic, triclinic, trigonal, hexanol). Their dividing is based on single side lengths and angles.

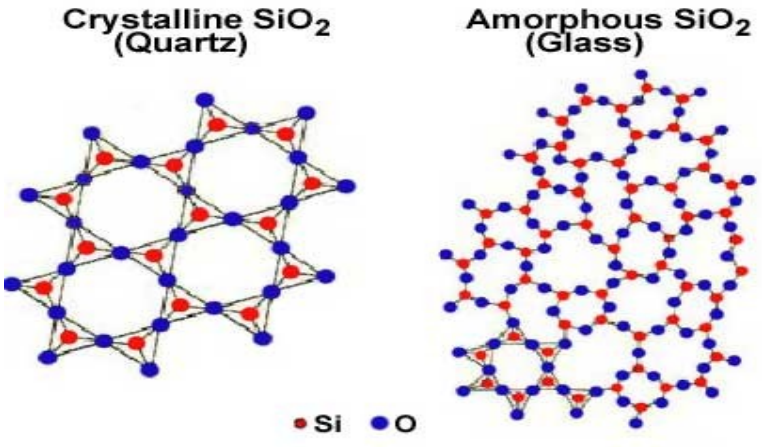
The crystalline state is usually more thermodynamically stable than amorphous one.

Amorphous state (3, 4)

For amorphous state it is sometimes used term glass as a synonymum. Glass is a physical state undergoing through a melt quenching or a glass transition. The term amorphous state can be used in general indefinite way.

It is a solid with a short-range molecular order and no long-range order of molecular packing or well-defined molecular conformation. They may posses residual crystalinity, polymorphic states. They can be characterized as the frozen solids and the cooled viscous liquid because of their random arranging of molecules. Because of the structure it is not possible to use X-ray diffraction.

Figure 1. Structural comparing of one compound (SIO_2) in crystalline and amorphous state (5)



Polymorphism (2, 3)

It is the ability of a solid material to have more than one possible crystal form. They can be different in physical and chemical properties and their transfer may have an influence on characteristics of final product. There exist several types of polymorphism; packing polymorphism (packing and bonding arrangement in different forms are different, conformational polymorphism, pseudopolymorphism (a new structure that is hydrated or solvated)

1. 1. 2 Amorphous solids; properties and preparation

Amorphous solids are used in many area of interest thanks to their properties. They are in polymers, ceramics, optical materials (glasses, fibers), foods, and also pharmaceuticals. They are the common form of certain materials (proteins, peptides, some sugars and polymers). The current interest has been elevated by two developments: a growing attention to pharmaceutical solids in general (especially polymorphs and solvates), a revived interest in the science of glass and the glass transition. (3)

Properties of amorphous solids

The amorphous solid properties are different from crystal form of the same substance. They are regulated by temperature.

Amorphous solids have higher solubilty, dissolution rate, and sometimes better compression characteristics than corresponding crystals. They are generally more unstable physically and chemically. They have higher energy, entropy and free energy than

corresponding crystals. (3)

Important characteristic is the glass transition temperature (T_g). The glass transition (6) is a reversible process when the amorphous, hard, glass like state goes to a rubber like state. T_g is a exact temperature where it is happenning. It is a kinetic parameter depending on temperature scanning rate, thermal history, and useful material descriptor. It is always lower than the melting temperature (T_m) of the crystalline state. Differential scanning calorimetry (DSC) can measure rate of possible crystallization and amount of present water that may have an influance on its stability and properties. DSC defines the glass transition as a change in the heat capacity as the amorphous solis goes from the glass state to the rubber state.

Preparation

We have two groups of materials good glass formers, where the preparation of amourphous solids is easy, and poor glass formers, where it is difficult.

We have several routes to prepare amorphous solids that include melt quenching, freeze-drying, spray-drying, rapid precipitation by addition of antisolvents, intruduction impurities, and solid dispersion.(3)

Melt quenching is not technique largely used for production of pharmaceutical products but it is very valuable in laboratory scale to prepare the amorphous state of compound. Solids are heated above the melting point and rapidly cooled down, typically by pouring into liquid nitrogen, to prevent a nucleation and growth. The amorphous form is trapped due to the continuously increasing viscosity of the rapidly cooling melt. In contrast, crystallization of melt occurs with an abrupt change in viscosity, during which crystallites grow in the body of the melt.(4)

Freeze-drying is a process for prepartion of stable material but can be rebuild by using water. It also used in food industry.

Spray-drying (7) is a technique to get amourphous solids by drying rapidly from solution. It must be done quickly to prevent possible recrystallize. Present of water leads to crystallize , so the amourphous solids must be kept in dry conditions.

1. 2 Drug Stability (8, 9, 10)

It is defined as capacity of a drug substance or drug product remaining in the specifications to maintain its identity, strength, quality and purity throughout the shelf-life. It is an important factor of safety, efficacy and quality of the product drug. If there is not of sufficient stability some changes in physical or chemical characteristics can happen. In the first case the drug can degrade to toxic derivatives, in the second case the drug may become esthetically unacceptable and in the last one it must be achieved that the oral formulation is enough stable under the pH conditions which are found in the gastrointestinal track.

The pharmaceutical products usually have a shelf-life of 3 years. During these years the potency should not decrease below 95% in the recommended conditions. The product should look and perform in the same way as the first time. The most common constant how to describe stability is the half-life. It is time at which the concentration has halved ($t_{1/2}$ or t_{50}). For the shelf-life it is expressed as t_{95} which means the time for 5% loss.

1. 2. 1 Chemical degradation (8)

There are several factors and conditions affecting chemical stability. Firstly it is a molecular structure of the drug. Other ones are temperature, pH, buffer species, ionic strength, light, oxygen, moisture, additives, and excipients.

Chemical drug degradation can be divided into five main groups as hydrolysis, dehydration, oxidation, isomerization and racemization, elimination, photolysis and complex interactions with excipients and other drug. Hydrolysis and oxidation are the most common pathways and then the photolysis.

Hydrolysis

It is the most likely cause of drug instability. We have two groups of solvents which can cause the hydrolysis. The first is water, it has a dominant role in dissolving. The second one is non-water solvent. In this case this process is called solvolysis.

Hydrolytic reactions involve nucleophilic attack of labile bonds, e.g. lactam > ester > amide > imide. There is number of conditions which can catalyse the breakdown: the presence of H_3O^+, OH^-, divalent ions, when ionic hydrolysis is faster than molecular, and

also heat, light, solution polarity and ionic strength and high drug concentration.

Many drugs are the most stable in range of pH 4 and 8. The extremes of pH can catalysed the degradation of the drugs. This is monitored by measuring degradation rates against pH, keeping, ionic strength, solvent concentration and temperature constant. We can also use acetate, lactate, phosphate, citrate or ascorbate buffers. Many drugs are unfortunately weak acids or basis. They have to be ionized for dissolution but it leads to more instability. It can be solve, in some cases, by addition a water-miscible solvent in the formulation.

Solvolysis is the term for breakdown when the reacting solvent is not water. This effect is connected with the polarity of the solvent. If a compound produces degradation products which are more polar then the addition of a less polar solvent will stabilize the formulation. If the degradation products are less polar, then the vehicle should be more polar to improve stability.

Dehydration

The dehydration is a chemical reaction that involves the loss of the water from the reacting molecule. Because the hydroxyl group (-OH) is a poor leaving group, having an Brønsted acid catalyst often helps by protonating the hydroxyl group to give the better leaving group, $-OH_2^+$. For example glucose and lactose undergo degradation to form 5-(hydroxymethyl)furural.

Oxidation

It is a loss of electrons and the oxidizing agent has to be able to take electrons. The reduction is the reverse reaction and there is a mutual exchange of electrons. Oxidation is called dehydrogenation in organic chemistry. The mechanism depends on the chemical structures of the drugs and the presence of reactive oxygen species or other oxidants. We can protect the drug by addition of antioxidants. For their successful it is necessary to use antioxidants which are more readily oxidized than the drug.

Isomerization and racemization

Isomerisation is the process by which one molecule is transformed into another molecule which has exactly the same atoms, but the atoms changed their places. Racemization is a partial conversion of one enantiomer into another. One of the most known examples of isomerisation are trans-cis isomerism, ketoaldose isomerism

Elimination

It is a chemical reaction where double bond is created. Atoms or group of atoms are eliminated from two neighboring carbon atoms. Between these two atoms double bond arises and so as a low molecular product. Dehydration is a subset of elimination. Other examples are decarboxylation, dehydrohalogenation.

Photolysis

Oxidation and some hydrolysis can be catalyzed by light. Increasing of energy can lead to decomposition, converting to heat, it can result in light emission at a new wavelength or be retained or transferred (10) We can protect drugs by using packaging into low actinic amber glass bottles, aluminium foil overwraps and blisters or cardboard outers.

Interactions with excipients and other drug

Quite often, reactions can occur between the drug and one or more additives. Similarly, two drugs might be formulated in the same product and react with each other. The example of these reacting drugs is reaction of amines with reducing sugars.

1. 2. 2 Physical degradation (8)

Drug substances together with excipients can exist in various microscopic physical states with different degrees of arrangement. Examples are amorphous and various crystalline, hydrated and solvated states. The components may change the state during the time from unstable to more stable. It all depends on chemical potential corresponding to the free-energy difference between the states and the energy barriers that must be overcome for the conversion.

Factors having an influence on physical degradation are the same as for chemical one, specifically plasticizing effect of water.

Crystallization of amorphous drugs

Drugs are often formulated as poorly water-soluble in their amorphous state. The ground is in their higher solubility in amorphous state than in their crystalline. However crystalline state has lower free energy leading to change of amorphous state into crystalline thermodynamically stable state within time. This may occur during long-term storage and can lead to many changes in their characteristics, clinical and toxicological behaviour.

Transitions in crystalline state

Polymorphs are various crystalline phases of the same drugs. They are different in their free energy and chemical potentials. All this depends on surrounding temperature and humidity. During the storage change of solubility and dissolution may occur. Differences can also exist between the anhydrous and hydrated forms of the same drug.

Formation and growth of crystals

Crystals in molecules do not remain static. They can increase, decrease, and travel across the medium which can be liquid or gaseous phase. The components may recrystallize or sublime onto the surface. This can be called “whisker” crystallization and may occur in porous tablets and at higher temperatures.

Vapor-phase transfer including sublimation

Also some components can sublime easily e.g. nitroglycerin. This was inhibited by using water-soluble fixing agents such as polyethylene glycol.

Moisture adsorption

This is mostly observed with solid pharmaceuticals. This can lead to changes in properties as appearance and dissolution rate.

1. 3 Techniques to improve stability

We have several techniques how to improve stability: the using cyclodextrins and formation of inclusion complexes, the incorporation into emulsiones, micelles and liposomes, the addition of stabilizers, the modification of molecular structure and the complex formation. (8) In following text I will focus more on cyclodextrins, liposomes, micelles, and emulsiones.

For some compounds it is necessary to arrange specific conditions during storing such as low temperature, no light and no access of oxygen. (8)

1. 3. 1 Cyclodextrins and formation of inclusion complexes (8, 11, 12, 13)

They belong to the family of cyclic oligosaccharides with a hydrophylic outer surface and a lipophilic central cavity. They were firstly isolated from starch in 1891 by a French scientist A. Villiers.

They consist of (α-1,4)-linked α-D-glucopyranose unit. They are in a chair formation causing shape like cones with secondary hydroxyl groups extending from the wider edge and the primary groups from the narrow edge. The naturally occurring cyclodextrins are α-CD containing 6 glucopyranose units, β-CD containing 7 units and γ-CD containing 8 units. They are displayed on the Figure 2. The real three-dimensional shape is displayed on the Figure 3. Except the naturally occurring CDs having quite low aqueous solubility there exist many CD derivatives resulting in dramatic improvement of their aqueous solubility. Examples of derived CDs: 2-Hydroxypropyl-β-cyclodextrin (HPβCD), Sulfonylether β-cyclodextrin sodium salt (SBEβCD), Randomly methylated β-cyclodestrin (RMβCD), 6-O-Maltosyl-β-cyclodextrin (G_2βCD), and 2-Hydroxypropyl-γ-cyclodextrin (HpγCD).

Figure 2. Structural representations of β-cyclodextrin, α-cyclodextrin, and γ-cyclodextrin (14)

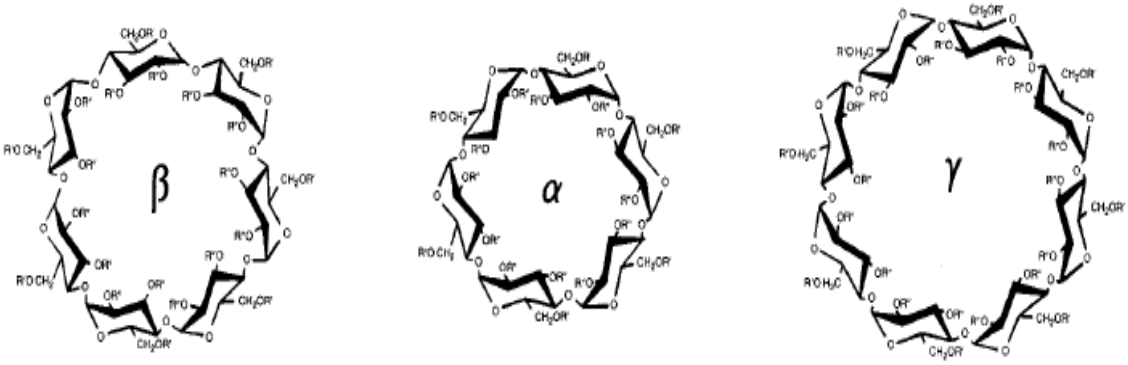
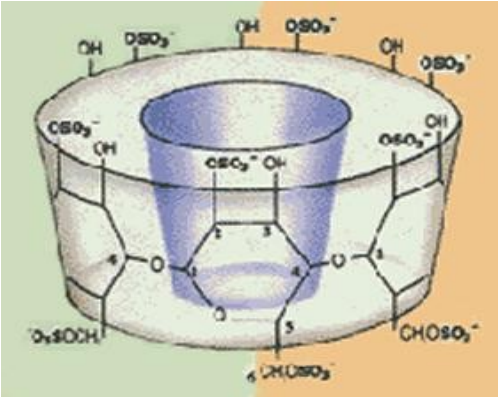


Figure 3. The real toroid shape of β-cyclodextrin (15)



Cyclodextrins have an ability to interact with poorly water-soluble drugs resulting in an increase in their apparent water solubility. The mechanism is based on formation of non-covalent dynamic inclusion complexes in solution. They can also form non-inclusion based complexes, aggregates, and form and stabilize supersaturated drug solutions. They are used as a carrier delivery system in the form of inclusion complexes with drug molecules. That has several advantages. It can enhance aqueous solubility, stability and biovailability, it can be used for prevention of incompatibility, masking of unpleasant taste and odour and reduction of irritation.

They are produced from starch by enzymatic conversion. There are used used two enzymes α-amylase and cyclodextrin glycosyltransferase (CGTase). As the first step starch is liquified by heating or using α-amylase then CGTase is added for the enzymatic conversion. It can synthesize all form of CDs. The product is a mixture in ratios depending on the enzyme used. The following purification is based on their water solubility.

Methods used for preparation of CD inclusion complexes are grinding, solid dispersion/co-evaporated dispersion, neutralization method, kneading method, precipitation method, spray drying, freeze drying and melting (11)

CDs can be widely used. They are used in pharmacy in many delivery systems such as oral, parenteral, ocular, nasal, rectal, dermal and transdermal delivery, and mostly used for lipid-soluble vitamins and hormones. They can be also used in food, and chemical industries, as well as agriculture and environmental engineering.

1. 3. 2 Incorporation into liposomes, micelles, and emulsiones

Liposomes (16, 17)

They are an artificially-prepared phospholipidic bilayer vesicles. Their size depends on the composition and preparation method. It is vary from 20 nm to 10μm. Thickness of bilayer membrane is 5nm. They can be divided into several groups according to to their structure and size.

SUV Small unilamellar vesicles 20 – 50nm

LUV Large unilamellar vesicles >50nm

OLV Oligolamellar vesicles 100nm – 1μm

MLV Multilamellar large vesicles 100nm – 10μm

MVV Multivesucullar vesicles 100nm – 10μm

They are composed of natural phospholipids and may also contain mixed lipids chains such as phosphatidylcholin, phosphatidylethanolamide, phosphatidylserin and phospatidylglycerol. The vesicle structures are displayed on the Figures 4a and 4b.

Figure 4a. Scheme of a liposome formed by phospholipids (18)

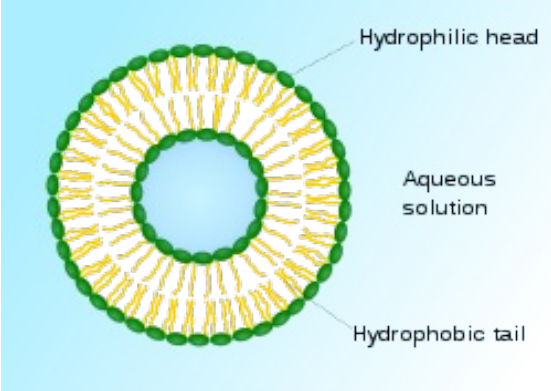
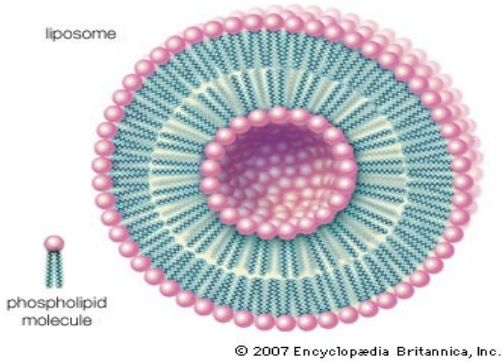


Figure 4b. Scheme of a liposome formed by phospholipids in three-dimension (19)



There are several methods to prepare liposome. (16) The mostly used is lipid hydration method. The others are solvent spherule method, sanitation method, solvent injection method, microfluidization method and freeze-thaw method.

They are used in pharmacy as carriers for drug molecules, both hydrophilic and hydrophobic. It can happened in three ways: encapsulation of hydrophilic molecules, adsorption of ambiphilic molecules and incorporation of hydrophobic molecules into the bilayer. Their advantages are reduction of toxicity and increasing of effectiveness. They can be target to the concrete spot in a body. They can modulate speed of release.

Liposomes can be used in dermal or transdermal preparations and in injections (amfotericine B, daunorubicine).

Except pharmacy they are used in agriculture, food industry and cosmetics.

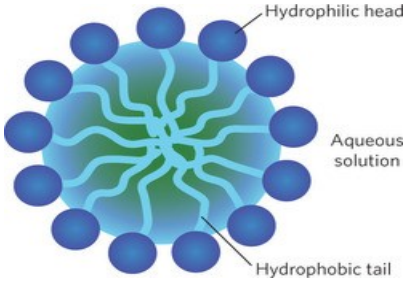
Micelles (20)

They are aggregates of surfactant molecules dispersed in a liquid. They have the ability as surfactants to low the surface tension. They consist two different parts; hydrophilic head and hydrophobic tail. In aqueous solution they form the aggregate where the head is oriented to the solution and the tail oriented to the centre of micelle showed at the Figure 5. We recognize two types of micelles – a normal phase micelle (o/w), and an inverse micelle (w/o). They can have various types of shapes – spherical, ellipsoids, cylinders, and bilayers. The side and shape depend on surrounding conditions such as surfactant concentration, pH, temperature, and ionic strength.

Important characteristic is the critical micelle concentration (CMC). It is the concentration where above that the micelles can only form and also temperature has to be greater than critical micelle temperature.

They are used as emulsifiers causing a compound, normally insoluble, soluble. In pharmacy they are important for absorption of fat-soluble vitamins and lipids in the human body. They are also part of detergents.

Figure 5. A normal phase micelle in aqueous solution (21)



Emulsiones (22)

They are mixtures of two or more misciblle liquids. In emulsiones, one liquid termed the dispersed phase, is disperesed in the other, termed the continuous phase. The phases are described according to phases in milk – water (w), and oil (o). We have several types of emulsiones, two basic; o/w miscible in water and w/o miscible in oil and some others; w/w, o/o, o/w/o, w/o/w.

Viscosity and color depend on the size of droplets. Viscosity is bigger with smaller droplets. During storage that gets lower with instability of droplets. The smaller one (under 0,1μm) are transparent. Particles bigger than 1μm are white.

They can also manifest several types of breakdowns – creaming, sedimentation, flocculation, phase inversion, coalescence, and Ostwald ripering. Creaming and sedimatation are results of gravitational or centrifugal forces. They are admissible in case of easy way to repair during using. Flocculation is a process of aggregation of the droplets into larger units. Phase inversion is a process of an exchange between the dispersed phase and the medium. It is useful property in preparations. Coalescence is the process of thinning and disruption of the liquid film between the droplets causing a formation of larger droplets. Ostwald ripering is a process of difuse transfer of small droplets in the biggers. That causes lower interface tension.

To ensure a stability of emulsiones we can do by using several techniques; an incresing of external phase viscosity by addition by e.g. glycerol, an increasing of internal phase viscosity, a droplet reducing, an enlargement of surfactant concentration and an increasing of internal phase ratio. These all techniques lead to lowering of surface tension or incresing viscosity.

The addition of emulsifiers (surfactants) is very important point during preparation. Their addition into the phase where are soluble is called English method. Their addition into the phase where are not soluble is called continental method. Other methods are a dilution of

emulsifiers by adding of both phases and in situ preparation.

There are stated applications of emulsiones: food industry – mayonnaise, salas creams, deserts; personal care, cosmetic products; pharmaceuticals – an anesthetics o/w emulsiones, lipod emulsiones, double and multiple emulsiones; paints.

1. 3. 3 The modification of molecular structure (8)

The goals of modification of molecular structure can be getting new molecules and elimination, improvement or change certain characteristics.

It is necessary to lower instability of some drugs. It is done by masking of reactive molecules. This type of modification improves stability in different pH conditions, more water-proof and it is useful againts enzyme degradation.

1. 3. 4 Complex formation (8)

Drugs and excipients formed into complexes can also improve stability. Between them there exist forces different nature. They can be van der Waals forces, dipole-dipole interactions, hydrogen bonding, Coulomb forces, and hydrophobic interactions. For stabilizations caffaine is often used together with e.g. benzocaine and procaine.

1. 3. 5 Addition of stabilizers (8)

This techniques lead to stabilization mostly againts oxygen, light and moisture. Effect of oxygen can be eliminated by using antioxidants. For this purpose it can be used α-tocoferol, butylated hydroxyanisole, ascorbic acid and thioglycolic acid.

Againts light, photodegration and moisture we use special photoprotective and moisture-proof films or in some cases oxygen absorbent. Photoprotective compounds are e.g. titanium dioxide, naphthylamine.

1. 4 Theoretical introduction to the experimental part

1. 4. 1 Chromatographic introduction

Chromatographic methods are widely used in analytical procedures. Their principle is separation of mixtures. They enable to measure both qualitative and quantitative information.

Chromatography (23, 24) uses to separation two phases. The first one is called the mobile phase and the second one is stationary phase. There is dissolved the mixture in the mobile phase, it also eluates single constituents from mixture and carries them at different speeds. The stationary phase has an ability to hold the constituents. There exist interaction between these two phases, causing their separation. There is forming repeatedly the dynamic balance between the sorption of stationary phase and the desorption to mobile phase during the procedure. The speed of eluation depends on how the constituents are held in the stationary phase.

Chromatographic methods can divide into several groups. The division can be done accoding to following characteritics: physical state of mobile phase (gas, liquid chromatography), chromatographic bed shape (column, paper, thin-layer chromatography), separation (adsorption, ion exchange, size-exclusion, affinity, distributive chromatography)

1. 4. 2 High Performance Liquid Chromatography

HPLC is one of the most common methods for identification, separation and quantification molecules.

It is a form of column chromatography. Its advantages are in simplicity and speed.

The equipment consists of a pumping system, a chromatographic column, an injector, a detector and a data acquisition system.(25) The mobile phase is supplied from one of several reservoirs and flows through the column, normally at a constant rate, and then through the detector.

The pumping system is capable to inject the solvents under pressure of 300 bars. Nowadays pressure systems are more efficient and they can operate with pressure up to 600 bars. The injection can be realized manually which can be inaccurate or by an auto-sampler.

We have several types of stationary phase. The splitting into these groups is based on mechanism of separation. We use silica, alumina or porous graphite in normal-phase chromatography, resins or polymers with acid or basic groups in ion-exchange chromatography, porous silica or polymers in size-exclusion chromatography, a variety of

chemically modified supports prepared from polymers, silica or porous graphite in reversed-phase liquid chromatography and finally special chemically modified stationary phase, e.g. proteins or peptides, cellulose or amylase derivatives.(25)

Mobile phase has to be clear and free from dissolved gases. For better separation of acid and basic compounds we can add a small amount of acids, alkalies, or buffer.

The separation (25) is based on interaction of compounds between stationary and mobile phase. The mechanism of retention is, when we use normal phase (silica material), adsorption by hydrogen bonds between the polar groups of the stationary phase and of the molecule. The molecules are washed in increasing polarity. In case of reversed-phase where the silica gel is usually coated with hydrocarbon chains of various lengths, normally used C4, C6 or C18 chains, the mechanism of retention is based on absorption by the Van der Waals interactions between the lipophilic regions of the molecules and the lipophilic chains of stationary phase. For example, those samples which have stronger connections with the mobile phase than with the stationary phase will elute from the column faster. It also means they have shorter retention time, while the reverse is also true.

There are several types of mobile phases: an isocratic elution - there is used constant amount and composition of mobile phase during the measurement, a gradient elution – there is used increasing the strength of the organic solvent.

HPLC is very useful method for identification molecules such as drug molecules, metabolites, lipids, peptides, etc. in analytical chemistry and biochemistry.

2. Experimental part

This experiment was realized on University of Eastern Finland, Faculty of Pharmacy. It was created at a lab of Deparment of Pharmaceutical Chemistry (DoPCh) but done for Department of Pharmaceutical Technology (DoPT) and their scietific project.

This is one of two diplomas created there. The first part of experiment is common for both works, the next parts are differ in used compounds but the procedure is same.

The final processing was done on Charles University, Faculty of Pharmacy in Hradec Králové.

My experiment was divided into three parts.

First part of experiment was focused on optimization of chromatographic conditions. It was used 38 active compounds (26) and there were tried various chromatographic conditions (various concentrations od mobile phase) The goal of this part was a determination of suitable mobile phase composition for the corresponding group of compounds.

Validation was done as the second step.

In the third last part there were done measurings if the compouds after melt quenching degrade or not. Standards and modified samples were compared.

2. 1 Instruments

Instrument used in the lab of DoPCh (using these instruments was a part of this work)

- HPLC equipment

Merck Hitachi LaChrom

Merck Hitachi Interface D-7000

Merck Hitachi Diode Array Detector L-7455

Merck Hitachi Programmable Autosampler L-7250

- Chromatographic column

HPLC Column Agilent Technologies

Zorbax Eclipse XDB-C18

Rapid Resolution HT 4,6x50mm 1,8-Micron 600 Bar

- Column oven (MetaChem Technologies Inc., California, USA)
- Analytical balance (Mettler Toledo AX205, Mettler Toledo, Greifensee, Switzeland)
- pH meter (Orion 3 Star Benchtop)

Instruments used in a lab of DoPT for a preparation of comperative samples (using these instruments was not a part of this work but it was worked with samples modified this way in experiment)

- Hot stage (Linkam Scientific Instruments Ltd, UK) of the polarizing light microscope (Nikon LV100D, Japan)
- Aluminium pans (Mettler Toledo, Switzerland)
- Analytical balance (Sartorius SE2, Sartorius AG, Germany)

2. 2 Chemicals

Chemicals for preparation of the mobile phase, the buffer and the solvent of active substances

HPLC methanol: HPLC Gradient Grade 99,8%, J.T.Baker, Deventer, The Netherlands

HPLC acetonitrile: HPLC Gradient Grade 99,9%, VWR Oy, Helsinki, Finland

Ortho-phosphoric acid 85%, Merck, Darmstadt, Germany

10mM solution of sodium hydroxide, FF-Chemicals, Haukipudas, Finland

purified and deionized water, Millipore Milli-Q system, Massachussets, USA

All used compounds:

Diflunisal, Sigma Aldrich, St. Louis, USA

Fenbufen, Sigma Aldrich, St. Louis, USA

Flurbiprofen, Sigma Aldrich, St. Louis, USA

Flutamide, Sigma Aldrich, St. Louis, USA

Ibuprofen, Sigma Aldrich, St. Louis, USA

Ketoprofen, Sigma Aldrich, St. Louis, USA

Mefenamic acid, Sigma Aldrich, St. Louis, USA

Naproxen, Sigma Aldrich, St. Louis, USA

Nimesulide, Sigma Aldrich, St. Louis, USA

Tolfenamic acid, Sigma Aldrich, St. Louis, USA

Acetazolamide, Sigma Aldrich, St. Louis, USA

Acetylsalicylic acid, Sigma Aldrich, St. Louis, USA

Benzocaine, Sigma Aldrich, St. Louis, USA

Captopril, Sigma Aldrich, St. Louis, USA

Chlorpropamide, Sigma Aldrich, St. Louis, USA

Chlorthiazide, Sigma Aldrich, St. Louis, USA

Cimetidine, Sigma Aldrich, St. Louis, USA

Clotrimazole, Sigma Aldrich, St. Louis, USA

Famotidine, Sigma Aldrich, St. Louis, USA

Furosemide, Sigma Aldrich, St. Louis, USA

Nitrofurantoin, Sigma Aldrich, St. Louis, USA

Nizatidine, Sigma Aldrich, St. Louis, USA

Paracetamol, Sigma Aldrich, St. Louis, USA
Perphenazine, Sigma Aldrich, St. Louis, USA
Piroxicam, Sigma Aldrich, St. Louis, USA
Primidon, Sigma Aldrich, St. Louis, USA
Pyrazinecarboxamid, Sigma Aldrich, St. Louis, USA
Pyridoxine, Sigma Aldrich, St. Louis, USA
Saccharine, Sigma Aldrich, St. Louis, USA
Salicylamide, Sigma Aldrich, St. Louis, USA
Salicylic acid, Sigma Aldrich, St. Louis, USA
Sulfadiazide, Sigma Aldrich, St. Louis, USA
Sulfadimidine, Sigma Aldrich, St. Louis, USA
Sulfamethoxazole, Sigma Aldrich, St. Louis, USA
Sulfamerazine, Sigma Aldrich, St. Louis, USA
Sulfanilamide, Sigma Aldrich, St. Louis, USA
Thiosalicylic acid, Sigma Aldrich, St. Louis, USA
Tolbutamide, Sigma Aldrich, St. Louis, USA

Purity was in a range of 97 and 101%.

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2. 3 Division of all used compounds according to their therapeutic applications

We had 38 active substances at the beginning. We divided them into several different groups. The first biggest group was non-steroidal anti-inflammatory drugs (NSAIDs) stated in the Table 1. There are 14 compounds. The second group was ATBs and chemotherapeutics also stated in Table 1. There are 7 compounds. In the Table 2 there were mentioned compounds belonging to the group of antidiabetics, diuretics, H2-receptor antagonists, local anaesthetics, food additives, anticonvulsive drugs, antiandrogens, antipyretics, antipsychotics, ACEis, antifungal drugs and vitamins. There are stated pKa values for every compound.

Table 1. The list of NSAIDs, ATBs and chemotherapeutics

NSAIDs	pKa	ATB + chemotherapeutics	pKa
acetylsalicylic acid	3,49	nitrofurantoin	7,20
diflunisal	2,94	pyrazinecarboxamide	13,91
fenbufen	5,60	sulfadiazine	6,50
flurbiprofen	4,14	sulfadimidine	7,75
ibuprofen	5,20	sulfamerazine	8,00
ketoprofen	4,23	sulfamethoxazol	5,81
mefenamic acid	4,20	sulfanilamide	10,10
naproxen	4,15		
nimesulide	8,49		
piroxicam	4,50		
salicylic acid	2,97		
salicylamide	8,37		
thiosalicylic acid	3,50		
tolfenamic acid	3,66		

Table 2. List of other used drugs

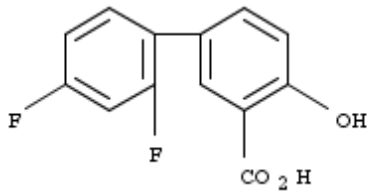
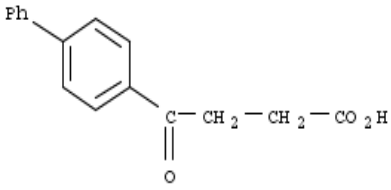
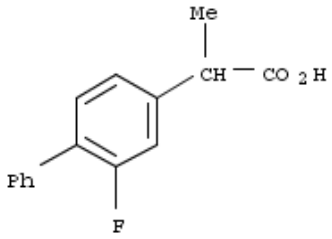
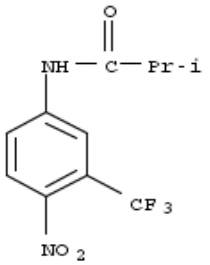
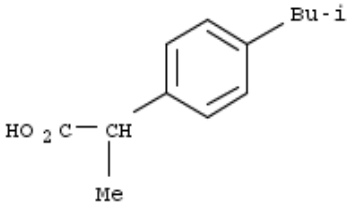
Therapeutical group	Compound	pKa
antidiabetics	chlorpropamide	5,00
	tolbutamide	5,16
diuretics	acetazolamide	6,80
	chlorthiazide	6,85
	furosemide	3,90
H2-receptor antagonists	cimetidine	6,80
	famotidine	7,75
	nizatidine	7,31
local anesthetic	benzocaine	2,51
food additive	saccharine	1,31
anticonvulsive drug	primidone	xx*
antiandrogen	flutamide	13,12
antipyretic	paracetamol	9,86
antipsychotic	perphenazine	7,94
ACEi	captopril	3,70
antifungal drug	clotrimazole	6,12
vitamine	pyridoxine	8,37

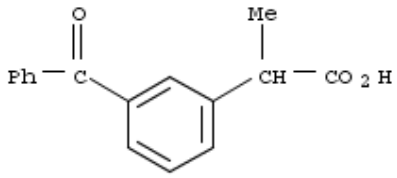
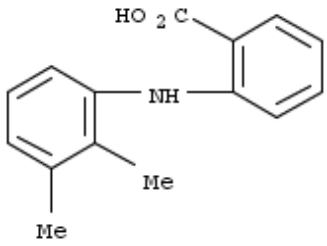
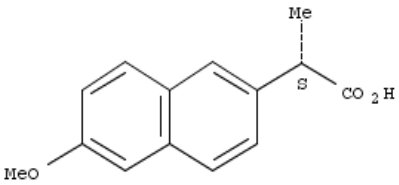
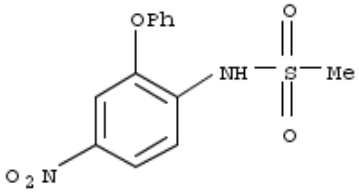
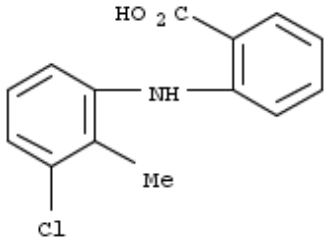
* xx – we did not find the value

2. 4 List and characterisation of chosen compounds used for validation and next comparing

There are stated some characteristics such as pKa value, molecular weight (M_r) and melting temperature (T_M) in ºC in Table 3. It also contains formulas of single compounds.

Table 3. Characterization of the compounds

Compound	Formula	
Diflunisal		pKa = 2,94±0,1 M_r = 250,20 T_M = 212,29±0,26
Fenbufen		pKa = 4,55±0,2 M_r = 254,28 T_M = 185,77±0,15
Flurbiprofen		pKa = 4,14±0,1 M_r = 244,26 T_M =113,95±0,21
Flutamide		pKa =13,1±0,7 M_r = 276,21 T_M = 111,84±0,03
Ibuprofen		pKa = 4,41±0,1 M_r = 206,28 T_M = 74,76±0,33

Compound	Formula	
Ketoprofen		pKa = 4,23±0,1 M_r = 254,28 T_M = 94,60±0,29
Mefenamic acid		pKa = 3,73±0,4 M_r = 241,29 T_M= 230,32±0,08
Naproxen		pKa = 4,84±0,3 M_r = 230,26 T_M = 155,33±0,08
Nimesulide		pKa = 5,93±0,1 M_r = 308,31 T_M = 148,64±0,05
Tolfenamic acid		pKa = 3,66±0,4 M_r = 261,7 T_M = 212,12±0,13

2. 5 The Preparations and HLPC Measuring

2. 5. 1 Preparation of mobile phase

The mobile phase was prepared by mixing ACN 90% and 50mM phosphate buffer in various ratios (concrete composition is stated in Table 4 in chapter 6. 1 Optimization of chromatographic method). It was degassed by helium about 5 minutes.

ACN 90% contained 90 volumes of acetonitrile and 10 volumes of water.

The buffer consisted 3,0 ml of 85% ortho-phosphoric acid added to 1000 ml water. It was finally adjusted to pH 2,1 with 10M solution of sodium hydroxide and filtered with 0,45 μm membrane (Durapore) and degassed with helium for about 5 minutes.

2. 5. 2 Preparation of samples for HPLC measuring

It was prepared standard stock solution at the beginning. It was dissolved 25 mg of the standard substance in methanol and diluted to 100 ml with the same solvent. The concentration of the stock solution was 0,25 mg/ml.

For samples it was added 0,8 ml of the stock solution to 9,2 ml of the 50mM phosphate buffer pH 2,1. In case of acidic labile substances such as tolfenamic acid and mefenamic acid it was used water instead of the buffer. The final concentration of the sample was 0,02 mg/ml.

For validation there were prepared two sets of stock solutions. Set No. 1 was for determination of precision, accuracy, linearity and stability and it was used for three days. Set No. 2 was freshly prepared after three days for comparing of stability.

2. 5. 3 Storage

The mobile phase and the buffer were storing in the room temperature, the stock solutions in refrigerator in 4°C and the samples were prepared before use.

2. 5. 4 Melt quenching (using this technique was not a part of this work but it was worked with samples modified this way in experiment)

The amorphous samples were heated from room temperature to 15°C above the melting point, and held there for 1 minute. Then they were cooled down as the next step to room temperature. The samples were analysed in sealed 40 μl aluminium pans with a pierced lid.

The weight of amorphous compounds on DSC pans was counted in the certain way to be comparable to the final concentration of standards in glass vial.

The sample was put in a 20ml volumetric flask and dissolved in methanol. The concentration of these stock solutions was about 0,25 mg/ml. Next step was done in the same way as non adjusted samples.

Stock solution concentrations of standards and modified samples are stated in Table 16 in part 2. 6. 3 Comparing of standards and modified amorphous samples.

2. 5. 5 HPLC Measuring

A Hitachi LaChrom apparatus consisted a L-7100 pump with programmable autosampler, a diode array detector and a MetaTherm column oven. It was under the control of D-7000 HPLC System Manager software.

For measurements it was used a reversed-phase C18,50 x 4,6 mm, 1,8 µm particle size column. Column oven was operated at 40°C. Detection of UV absorbance was monitored at 214, 222, 228, 254 nm. The injection volume was 5 µl. Total running time was 6 min.

3. Reasults and discussion

The main target of this experiment was to develop right method for several compounds to measure their stability and concetration if they after heating and cooling down degradate. Every standard coumpound was measured individually to gain pure spectrum with no disturbing peaks. The goal was not identify the possible degradents.

3. 1 Optimization of chromatographic method

They were determinated several conditions for choosing right method. It was also necessary to gain spectra and download them to the library of HPLC equipment for final comparing of stability. These compounds are listed in the part Chemicals.

All coumpounds have different pKa values, structure, molecular weight and UV spectra. Because all compounds are ionic so buffer had to be used.

Chromatographic conditions

Column: a reversed-phase C18, 50 x 4,6 mm, 1,8µm particle size.

Mobile phase: Solvent A: ACN 90%

Solvent B: 50mM phosphate buffer pH 2,1

Temperature: 40°C

Flow rate: 1ml/ml

Injection volume: 5µl (c=0,02mg/ml)

Running time: 10 minutes

For getting speed of eluation there were done several experiments with different ratios of ACN 90% and the buffer. These ratios are stated in Table 4. 1st method was done with all compounds to get spectra and maximum absorbance wavelengths. First three methods served as separation method to find their properties in these ratios. Due to measured results of three first method, 5 others were tested specifically for only several compounds to get the best ratio.

Selective conditions

Separation time (Rt running time): 5 – 10 minutes

Resolution (k capacity factor): 1,0 – 10,0

Peak height: large S/N ratio

Pressure: less than 150 Bar

Table 4. Ratio list of ACN and buffer

	Contents	
	ACN 90% (ml)	buffer pH 2,1 (ml)
1st method	65	35
2nd method	50	50
3rd method	30	70
4th method	60	40
5th method	45	55
6th method	57	43
7th method	40	60
8th method	35	65

All compounds were dissolved in methanol. Concentration of stock solutions were 0,25 mg/ml. pKa range of used compounds was wide. It was not possible to get one method for all. For separation was used a reversed-phase column and isocratic eluation. It was important to find right ratio of mobile phase and the buffer to have capacity factors in set interval.

All compounds were measured by a diode-array detector and there were monitored multiple wavelengths.

In following Table 5 there are stated all compounds with measured Rt.

Table 5. List of Rt all compounds

	Rt (min)							
Compounds	1st	2nd	3rd	4th	5th	6th	7th	8th
Acetazolamide	0,49	0,52	0,62	X	X	X	X	X
Acetylsalicylic acid	0,59	0,71	1,89	X	X	4,49	0,90	1,07
Benzocaine	0,73	1,03	2,53	X	X	X	1,47	1,88
Captopril	0,53	0,59	0,87	X	X	X	X	X
Chlorpropamide	0,77	1,25	5,39	X	X	X	2,24	1,53
Chlorthiazide	0,49	0,54	0,70	X	X	X	X	X
Cimetidine	0,43	0,43	0,47	X	X	X	X	X
Clotrimazole	0,65	0,98	X	X	X	X	2,08	3,77
Diflunisal	1,11	2,73	X	1,39	6,29	1,71	X	X

	Rt (min)							
Compounds	1st	2nd	3rd	4th	5th	6th	7th	8th
Famotidine	0,43	0,43	0,47	X	X	X	X	X
Fenbufen	0,95	1,89	X	1,09	4,09	1,30	X	X
Flurbiprofen	1,26	3,11	X	1,58	6,11	1,95	X	X
Flutamide	1,38	3,50	X	1,75	6,52	2,18	X	X
Furosemide	0,60	0,88	3,44	X	1,77	X	1,45	2,14
Ibuprofen	1,67	4,43	X	2,15	7,36	2,65	X	X
Ketoprofen	0,89	1,65	X	1,05	2,84	1,19	3,51	5,92
Mefenamic acid	2,17	6,54	X	3,01	10,00	3,73	X	X
Naproxen	0,92	1,73	X	1,08	2,78	1,23	3,65	6,16
Nimesulide	1,10	2,53	X	X	X	1,67	X	X
Nitrofurantoin	0,53	0,60	0,98	X	X	X	X	X
Nizatidine	0,43	0,43	0,46	X	X	X	X	X
Paracetamol	0,50	0,31	0,59	X	X	X	X	X
Perphenazine	0,53	0,65	2,96	X	X	X	1,00	1,53
Piroxicam	0,86	1,29	4,17	X	X	X	2,04	2,78
Primidon	0,51	0,59	0,83	X	X	X	X	X
Pyrazincarboxamide	0,48	0,49	X	X	X	X	X	X
Pyridoxin	0,43	0,43	0,45	X	X	X	X	X
Saccharin	0,47	0,51	0,60	X	X	X	X	X
Salicylamide	0,57	0,65	1,03	X	X	X	X	X
Salicylic acid	0,63	0,84	1,35	X	1,41	X	1,15	1,45
Sulfadiazine	0,51	0,55	0,71	X	X	X	X	X
Sulfadimidine	0,55	0,62	0,89	X	X	X	X	X
Sulfamethoxazole	0,57	0,71	1,39	X	X	X	0,91	1,11
Sulfamerazine	0,53	0,59	0,79	X	X	X	X	X
Sulfanilamide	0,49	0,52	X	X	X	X	X	X
Thiosalicylic acid	0,37	0,89	2,11	0,71	1,43	X	1,25	X
Tolbutamide	0,85	1,50	7,86	0,98	2,23	X	2,95	4,65
Tolfenamic acid	2,50	8,19	X	0,71	X	4,49	X	X

X – it was not measured

Based on the measured values of Rt (Table 5) and the conditions mentioned above as the best method for my next work was chosen the 6th one with ratio of ACN 90% and the buffer 57:43. This method was suitable for ten following substances – diflunisal, fenbufen, flurbiprofen, flutamide, ibuprofen, ketoprofen, mefenamic acid, naproxen, nimesulide and tolfenamic acid. All these compounds except flutamide are NSAIDs with pKa around 3 – 5. Flutamide is antiandrogen with pKa 13. We could not explain how this compound would be within set conditions.

In following Table 6 there are stated numbers of suitable compounds for each method. Althought 5th method has the biggest number the Rt of compounds in interval they are too big. From technical reason 6th method was chosen with smaller number of compounds.
8th method was chosen for the second thesis.

Table 6. Number of suitable compounds for each method

Method	Number of suitable compounds
1st method	3
2nd method	9
3rd method	10
4th method	5
5th method	11
6th method	10
7th method	7
8th method	8

3. 2 Validation process

Validation was designed and performed according to ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Text and Methodology Q2(R1). (27) During the experiment it was used the assay as a type of analytical procedure. Based on this methodology accuracy, precision – repeatability, intermediate precision, specificity, linearity and range were done. Detection limit and quantitation limit were not assessed they are not normally evaluated for this type of experiment. Also robustness and reproducibility were not assessed because it was not necessary to transfer, repeat and verify following results in another laboratory. It was performed system suitability test before measurings.

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3. 2. 1 Specificity

It is defined as the ability to measure accurately the concentration of an analyte in the presence of all other sample materials. (28)

There were formulated some acceptance criteria. The first one was capacity factor k between 1 and 10. The second one was no disturbing peaks at retention time of analyte. The third one was peak pure according peak purity test and similarity of spectrum was over 95% compared to the library spectrum. They are stated in Table 7.

Results were measured in maximum absorbance wavelenghts. They are also stated in the following Table 7.

Table 7. List of capacity factors and purity of single compounds

	Rt (min)	k	purity	Wavelenght(nm)
Diflunisal	1,50	2,2	1,0000	228
Fenbufen	1,25	1,7	0,9998	214
Flurbiprofen	1,85	2,9	0,9997	222
Flutamide	2,12	3,5	1,0000	228
Ibuprofen	2,58	4,5	1,0000	214
Ketoprofen	1,17	1,5	0,9999	222
Mefenamic acid	3,59	6,6	1,0000	222
Naproxen	1,21	1,6	0,9997	228
Nimesulide	1,55	2,3	1,0000	214
Tolfenamic acid	4,29	8,1	1,0000	222
Void volume	0,47			

3. 2. 2 Linearity

It is a measure of how well a calibration plot of response versus concentration approximates a straight line. (28)

For measurements there were prepared samples with 25, 50, 75, 100 and 125% of the target concentrations (0,02 mg/ml). The stock solutions were used for preparing corresponding dilutions stated in Table 8. It was used 10ml volumetric flask for mixing the stock solution with buffer or water.

Table 8. The ratios between stock solution and the buffer in the certain concentrations

percent	concentration (mg/ml)	ml of stock solution	ml of buffer/water
25	0,005	0,2	9,8
50	0,010	0,4	9,6
75	0,015	0,6	9,4
100	0,020	0,8	9,2
125	0,025	1,0	9,0

This procedure was repeated three times.

Linearity was evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. There should be a linear relationship in an ideal situation expressed by a straight line. Test results were evaluated by calculating of a regression line by the method of least squares. There were also calculated correlation factor; value above 0,997 was accepted, y-cutting (intercept) ≐ 2% from 100% sample area, response factor less than ± 2,5 % from 100% and finally slope of the regression line and residual sum of squares stated in Tables 9 and 10.

Table 9. List of measured and counted y-intercepts, slope of regressions, border values (probability 95%), correlation coefficient and y-cutting

T**Diflunisal**	Coefficients	Lower 95%	Upper 95%
y-intercept	17607,9	-138225	173441
slope of regression	951,5	-899	2802
correlation coefficient	0,0876		
y-cutting (%)	7,70		
Fenbufen	Coefficients	Lower 95%	Upper 95%
y-intercept	28296,2	7794	48799
slope of regression	633,9	393	875
correlation coefficient	0,7126		
y-cutting (%)	32,50		
Flurbiprofen	Coefficients	Lower 95%	Upper 95%
y-intercept	8533,5	6366	10701
slope of regression	476,9	451	502
correlation coefficient	1,0000		
y-cutting (%)	14,20		
Flutamide	Coefficients	Lower 95%	Upper 95%
y-intercept	15645,9	14045	17247
slope of regression	744,8	726	754
correlation coefficient	1,0000		
y-cutting (%)	17,20		
Ibuprofen	Coefficients	Lower 95%	Upper 95%
y-intercept	13338,2	10605	16071
slope of regression	591,7	559	624
correlation coefficient	1,0000		
y-cutting (%)	17,90		
Ketoprofen	Coefficients	Lower 95%	Upper 95%
y-intercept	13965	12210	15720
slope of regression	733,7	713	755
correlation coefficient	0,9977		
y-cutting (%)	16,40		

Mefenamic acid	Coefficients	Lower 95%	Upper 95%
y-intercept	58554,2	9132	107977
slope of regression	1015,8	435	1597
correlation coefficient	0,5233		
y-cutting (%)	39,10		
Naproxen	Coefficients	Lower 95%	Upper 95%
y-intercept	106022,5	93392	118653
slope of regression	5259,6	5111	5408
correlation coefficient	1,0000		
y-cutting (%)	16,50		
Nimesulide	Coefficients	Lower 95%	Upper 95%
y-intercept	43878,4	23956	63801
slope of regression	456,6	218	695
correlation coefficient	1,0000		
y-cutting (%)	40,10		
Tolfenamic acid	Coefficients	Lower 95%	Upper 95%
y-intercept	49008,7	32557	65460
slope of regression	1327,9	1129	1527
correlation coefficient	1,0000		
y-cutting (%)	25,40		

Generally speaking based on these results we can say this measuring did not go well. Border values are too wide. Correlation coefficients of diflunisal, fenbufen and mefenamic acid are too low but that does not mean there is no linear relationship. Y-cutting does not comply with stated interval in all cases.

Table 10. Response factors and exact concentrations

Compound	Concentration (%)	Responce factor (%)	Compound	Concentration (%)	Responce factor (%)
Diflunisal	25,39	155,9	Ketoprofen	24,97	66,0
	50,78	220,0		49,94	84,3
	76,17	226,1		74,91	91,3
	101,56	100,0		99,88	100,0
	126,95	412,6		124,85	100,0
Fenfufen	25,63	54,2	Mefenamic acid	25,65	42,0
	51,26	72,3		51,30	56,9
	76,89	72,9		76,95	109,8
	102,52	100,0		102,60	100,0
	128,15	100,8		128,25	89,0
Flurbiprofen	25,50	72,2	Naproxen	25,59	65,9
	51,00	92,1		51,18	86,3
	76,50	101,9		76,77	95,2
	102,00	100,0		102,36	100,0
	128,15	109,8		127,95	102,9
Flutamide	25,40	65,6	Nimesulide	25,20	60,0
	50,80	85,1		50,40	78,1
	76,20	94,0		75,60	98,1
	102,00	100,0		100,80	100,0
	127,00	102,8		126,00	164,0
Ibuprofen	25,23	67,6	Tolfenamic acid	24,91	72,2
	50,46	87,1		49,82	92,1
	75,69	94,7		74,73	101,9
	100,92	100,0		99,64	100,0
	126,92	108,0		124,55	109,8

The most of gained values are not also inside the interval. In 25% and 50% concentration it is not so important it was not measured with these low concentrations further but deviations from set 2,5% are too high. In higher concentrations they are mostly closer except diflunisal that is completely out of interval.

3. 2. 3 Range

It is the lower and upper concentrations for which the analytical method has adequate accuracy, precision, and linearity. (28)

In this case the range was between 25 and 125%.

3. 2. 4 Accuracy

It is the closeness of the measured value to the true value. (28)

It was assessed by using 9 different determinations over 3 concentration levels covering the specified range, for this case that was 3 replicates for every concentration. Values are averaged. Accuracy was calculated as a ratio of concentration measured and concentration weighted multiply 100. The results are stated in Table 11.

Table 11. Accuracy

Compound	Concentration (%)	Concentration weighted (µg/ml)	Concetration calculated (µg/ml)	Accuracy (%)
Diflunisal	76,17	15,24	9.83	64,5
	101,56	20,32	44,40	218,5
	126,95	25,39	10,84	42,7
Fenbufen	76,89	15,27	19,23	125,9
	102,52	20,45	18,51	90,5
	128,15	25,63	25,14	98,1
Flurbiprofen	76,50	15,40	15,02	97,5
	102,00	20,45	21,60	105,6
	127,50	25,50	25,01	98,1
Flutamide	76,20	15,24	15,27	100,2
	101,60	20,32	20,20	99,4
	127,00	25,40	25,48	100,3
Ibuprofen	75,69	15,30	15,65	102,3
	100,92	20,18	20,72	102,7
	126,15	25,23	24,70	97,9
Ketoprofen	74,91	14,99	15,29	102,0
	99,88	19,98	19,44	97,3
	124,85	24,97	24,70	101,0

Compound	Concentration (%)	Concentration weighted (μg/ml)	Concetration calculated (μg/ml)	Accuracy (%)
Mefenamic acid	76,95	15,29	8,58	56,1
	102,60	20,47	17,93	87,6
	128,25	25,65	29,75	116,6
Naproxen	76,77	15,25	15,14	99,3
	102,36	20,57	20,53	99,8
	127,95	25.59	25,69	100,4
Nimesulide	75,60	15,12	10,4	114,2
	100,80	20,16	21,55	142,5
	126,00	15,12	17,34	68,8
Tolfenamic acid	74,73	14,95	0,58	3,9
	99,64	19,93	0,78	3,9
	124,55	24,91	0,58	3,4

Accuracy of flurbiprofen, flutamide, ibuprofen, ketoprofen and naproxen goes to 100% in all three tested concentrations. Fenbufen has bigger deviation. Results for diflunisal, nimesulide and tolfenamic acid are very inaccurate.

3. 2. 5 Precision

It is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. (28) It is divided into three groups; repeatability – the precision under same operating conditions over a short period of time, intermediate precision – the agreement of complete measurements in the same laboratory during different days, and reproducibility – the precision between laboratories.

For our purpose there were used just first two types.

Every sample was measured three following days in six replicates in 100% (0,02 mg/ml) concentration.

Precision was expressed by using relative standard deviation (RSD) and precision confidence interval (CI). RSD under 10% was accepted. Table 12 is for set No. 1, Table for 13 is for set No. 2

Table 12. Precision, set No. 1

Compound	SD of area	RSD of area (%)	Area	CI
Diflunisal	48907	41,99	116478	± 513132
Fenbufen	24419	16,62	146922	±25620
Flurbiprofen	1025	1,68	61054	±1075
Flutamide	757	0,83	91610	±794
Ibuprofen	1863	2,49	74783	±1956
Ketorpofen	737	0,87	84906	±773
Mefenamic acid	11709	9,92	118083	±12285
Naproxen	8331	1,28	649076	±8741
Nimesulide	29310	25,97	112883	±30752
Tolfenamic acid	6524	4,74	37510	±66845

Table 13. Precision, set No. 2

Compound	SD of area	RSD of area (%)	Area	CI
Diflunisal	108990	79,34	137371	±114352
Fenbufen	5414	2,96	182594	±5680
Flurbiprofen	1161	1,99	58412	±1218
Flutamide	1313	1,56	87584	±1378
Ibuprofen	1581	2,04	77522	±1659
Ketoprofen	512	0,62	83526	±537
Mefenamic acid	11601	5,04	230457	±12172
Naproxen	9776	1,60	609703	±10257
Nimesulide	4756	4,42	107726	±4990
Tolfenamic acid	21434	9,81	218492	±22489

Results from set No. 2 are better. All compounds except diflunisal fulfil criterion. In case of set No. 1 there 3 compounds with very bad results and mefenamic acid has border values.

3. 2. 6 Stability

For stability studies there were compared results from the second day of precision and the third day of precision with freshly prepared stock solution. If stability of the solution was low, the next analyses were performed during one day. The results are stated in Table 14.

Table 14. Stability of compounds during 3 days

	Stability (%)		
Compound	1st day	2nd day	3rd day
Diflunisal	188,50	184,1	204,3
Fenbufen	80,46	16,2	7,9
Flurbiprofen	104,52	102,7	102,5
Flutamide	104,60	102,7	105,5
Ibuprofen	96,47	96,0	99,0
Ketoprofen	101,66	99,9	104,1
Mefenamic acid	51,24	36,1	20,8
Naproxen	106,46	78,4	70,0
Nimesulide	104,79	30,4	25,3
Tolfenamic acid	62,11	46,9	41,3

Flurbiprofen, flutamide, ibuprofen, ketoprofen are stable compounds. Naproxen is unstable. Its solutions must be prepared freshly before experiment. Results of diflunisal, fenbufen, nimesulide, mefenamic acid and tolfenamic acid are ambiguous. They cannot be used as a relevant. Generally tolfenamic and mefenamic acids are unstable in acidic solutions. Solutions for next experiment were prepared right before measuring.

3. 2. 7 System suitability test

Every day before measurements there was SST. It was done by using ketoprofen as a stable substance with six replicates. It was calculated RSD of Rt and the area under the peak. The criterion was less than 1%. RSD was in every cases under 1%.

3. 2. 8 Re-measuring

Based on previous unsatisfactory results there was a discussion. Almost every results of diflunisal, mefenamic acid, naproxen, nimesulide and tolfenamic were out of interval or nonsense. After that we found that mistake was in using still same plastic pipettes. They could not take exactly same volume for whole experiment. It was designed one other measuring for confirmation of this hypothesis. It was used only glass pipette.

It was used lineary test again with ketoprofen as the most stable compound.

It was prepared 1 basic stock solution by dissolving approximately 25 mg of ketoprofen in methanol and diluting to 100 ml with the same solvent (Table 15a). From this stock

solution it was prepared Dilution I. 10 ml of stock solution was taken and put into 50ml volumetric flask and solved in mobil phase. The concentration of this dilution was approximately 0.05 mg/ml (Table 15b). From this dilution there were prepared 5 others dilutions (standard = STD) (Table 15c). From STD 2 there were prepared 3 other dilutions (Table 15d). Every standard was measured in three replicates. Exact values are stated in the following tables.

Table 15 a) Preparing of stock solution

Ketoprofen	Weight (mg)	Concentration (mg/ml)	Calculated concetration (mg/ml)
Stock solution	24,98	0,2500	0,2498

Table 15 b) Preparing of Dilution I

	Volume from stock solution (ml)	Volume dilution (ml)	Concentration (mg/ml)	Calculated concetration (mg/ml)
Dilution I	10,00	50,00	0,0500	0,0500

Table 15 c) Dilutions from Dilution I

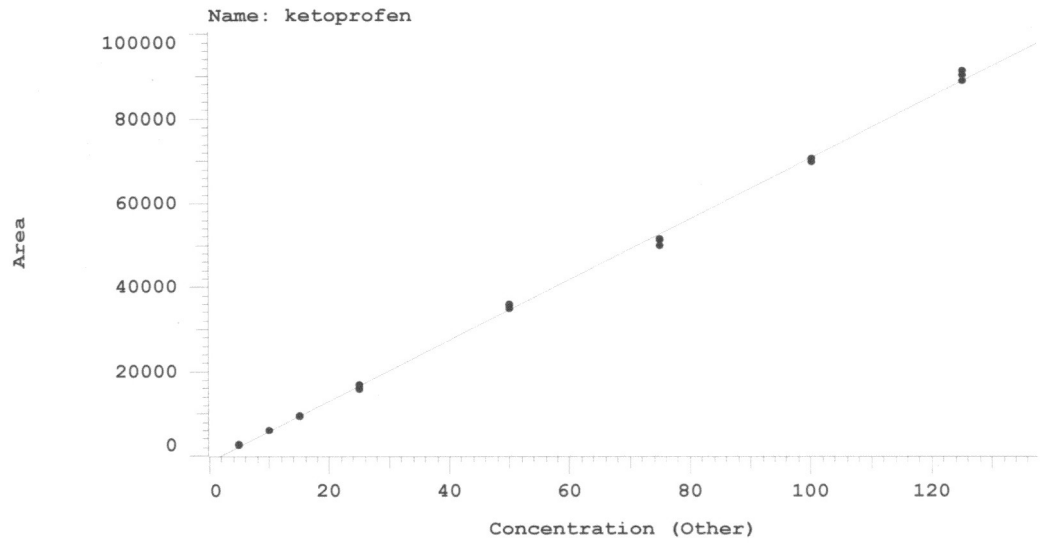
Standard	Volume from Dilution I (ml)	Dilution Volume (ml)	Concentration (mg/ml)	Calculated concentration (mg/ml)	Calculated concentration (%)
STD1	1,00	10,00	0,0050	0,004996	24,98
STD2	2,00	10,00	0,0100	0,009992	49,96
STD3	3,00	10,00	0,0150	0,014988	74,88
STD4	4,00	10,00	0,0200	0,019984	99,92
STD5	5,00	10,00	0,0250	0,024980	124,90

Table 15 d) Dilutions from STD2

Standard	Volume from Dilution I (ml)	Dilution Volume (ml)	Concentration (mg/ml)	Calculated concentration (mg/ml)	Calculated concentration (%)
STD6	1,00	10,00	0,0010	0,0009921	4,97
STD7	2,00	10,00	0,0020	0,0019984	9,99
STD8	3,00	10,00	0,0030	0,0029976	14,99

Following graph shows how exact was measuring. Calculated concentrations and measured concentrations are almost identical. Y-intercept is getting closer to zero.

Graph 1. Repeated linearity test with ketoprofen



According these results we can say that the mistake was really in using plastic pipettes. During third part there was used only glass pipette and solutions were prepared right before measuring to prevent another inaccuracy.

Although results from validation were very inaccurate we decided to continue in our experiment and not to repeat it. Final results are not impacted.

3. 3 Comparing of standards and modified samples

In the last part of experiment we studied stability of compounds after heating and cooling if they degraded or not. We measured again freshly prepared standard solutions of 10 chosen compounds and modified amorphous samples prepared by laboratory of Department of Pharmaceutical Technology. Weight was calculated to be comparable to each other. In Table 16 there are exact concentrations of stock solutions.

Table 16. Concentrations of standards and modifies samples in stock solutions

	Calculated concentration (mg/ml)	
	Standards	Modified samples
Diflunisal	0,2490	0,2497
Fenbufen	0,2749	0,2754
Flurbiprofen	0,2635	0,2673
Flutamide	0,2863	0,2847
Ibuprofen	0,2700	0,2692
Ketoprofen	0,2863	0,2849
Mefenamic acid	0,2015	0,2039
Naproxen	0,2930	0,2949
Nimesulide	0,2684	0,2664
Tolfenamic acid	0,2307	0,2254

In the following Table 17 there are results for every single compound. The most important factors for comparing are purity, should be >0,9997, and stability <5%. RSD <2,5% is accepted.

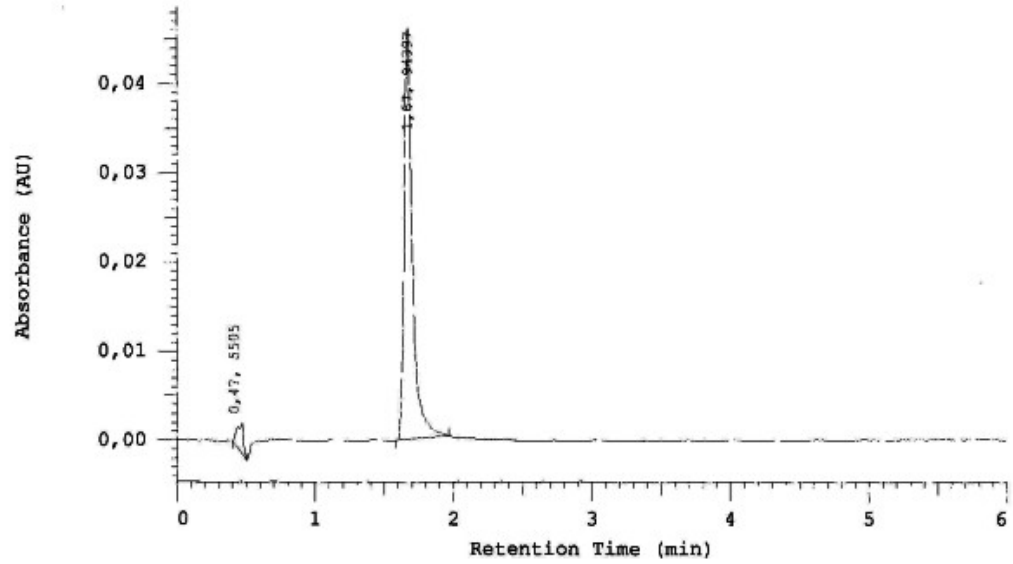
Table 17. Comparing standards and samples after melt-quenching

NSAIDs				
compounds	Rt	conc (mg/ml)	Stability (%)	Purity
diflunisal	1,67	0,0278	111,3	0,9998
RSD(%)	0,35	0,87	0,87	0
fenbufen	1,17	0,0257	93,3	0,9993
RSD(%)	0,00	0,3	0,3	0,01
flurbiprofen	1,94	0,0279	104,4	0,9997
RSD(%)	0,52	1,16	1,16	0,01
ibuprofen	2,70	0,0275	102,2	1,0000
RSD(%)	0,43	0,69	0,69	0
ketoprofen	1,13	0,0291	102,1	0,9996
RSD(%)	2,85	0,64	0,64	0,01
mefenamic acid	3,71	0,0199	97,6	0,9999
RSD(%)	0,27	0,81	0,81	0
naproxen	1,18	0,0297	100,7	0,9998
RSD(%)	0,49	0,8	0,8	0
nimesulide	1,65	0,0278	92,9	0,9997
RSD(%)	0,35	0,98	0,98	0,01
tolfenamic acid	4,47	0,0229	101,6	0,9999
RSD(%)	0,59	4,39	4,39	0
antiandrogen				
flutamide	2,07	0,0273	95,9	0,9997
RSD(%)	0,28	1,17	1,17	0

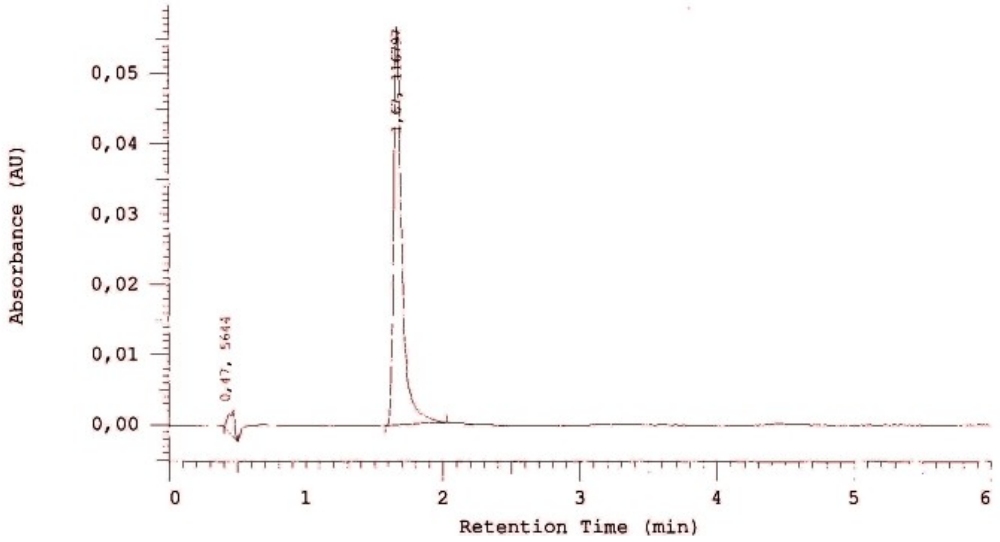
Purity of compounds is high in every cases. Diflunisal, fenbufen and nimesulide are outside of the interval but not so much.

For better comparing there are shown below representative chromatograms of every standards and amorphous samples

Figure 6. Representative chromatograms of diflunisal std (A) and diflunisal sample (B) with Rt and area

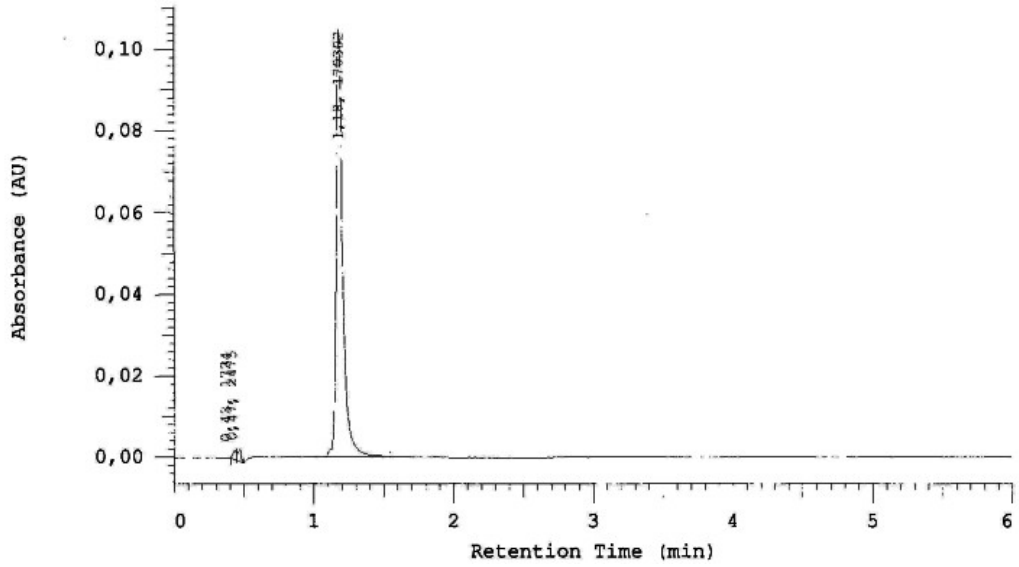


A: Rt = 1,67, Area = 103916

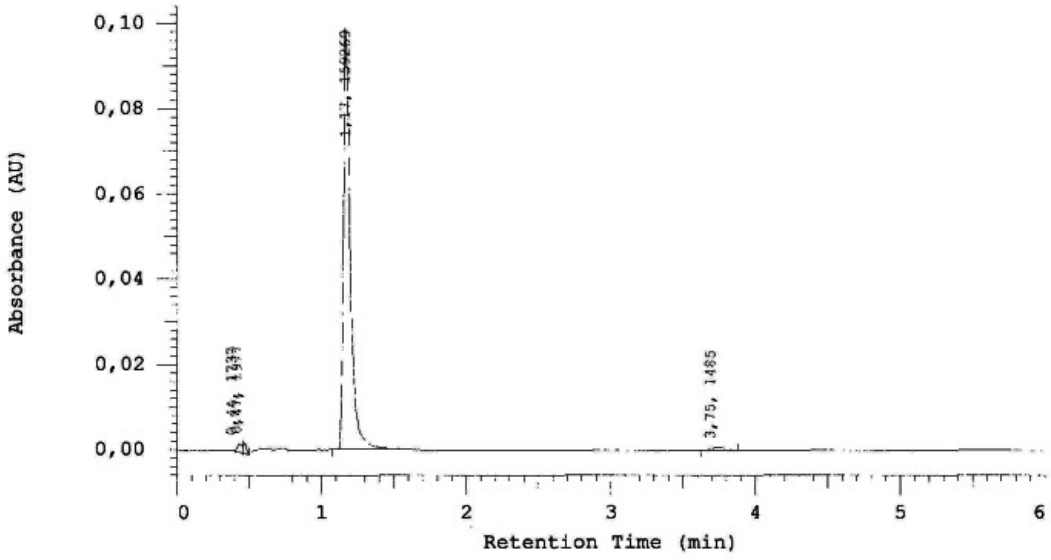


B: Rt = 1,67, Area = 116204

Figure 7. Representative chromatograms of fenbufen std (C) and fenbufen sample (D) with Rt and area

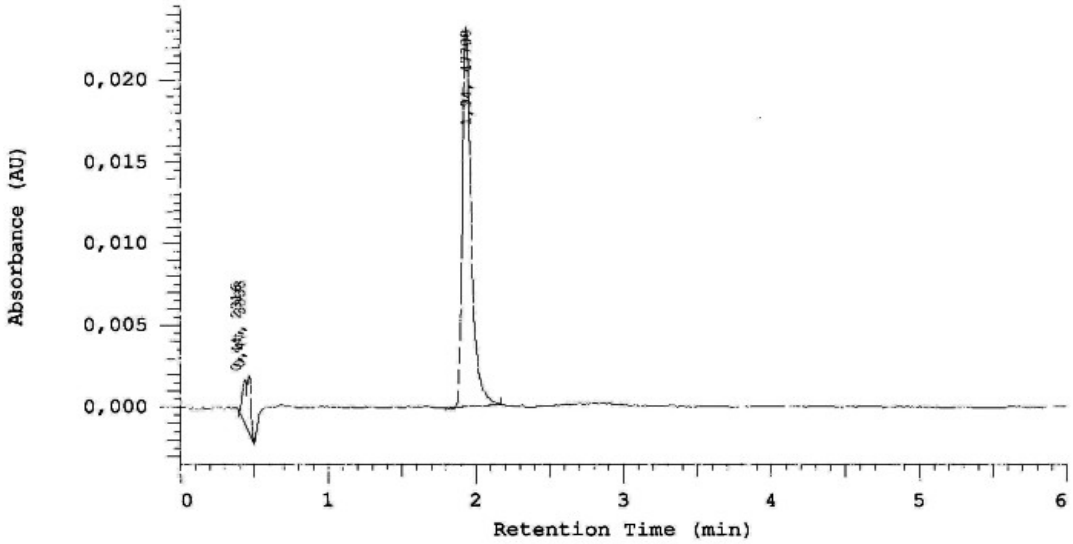


C: Rt = 1,18, Area = 169965

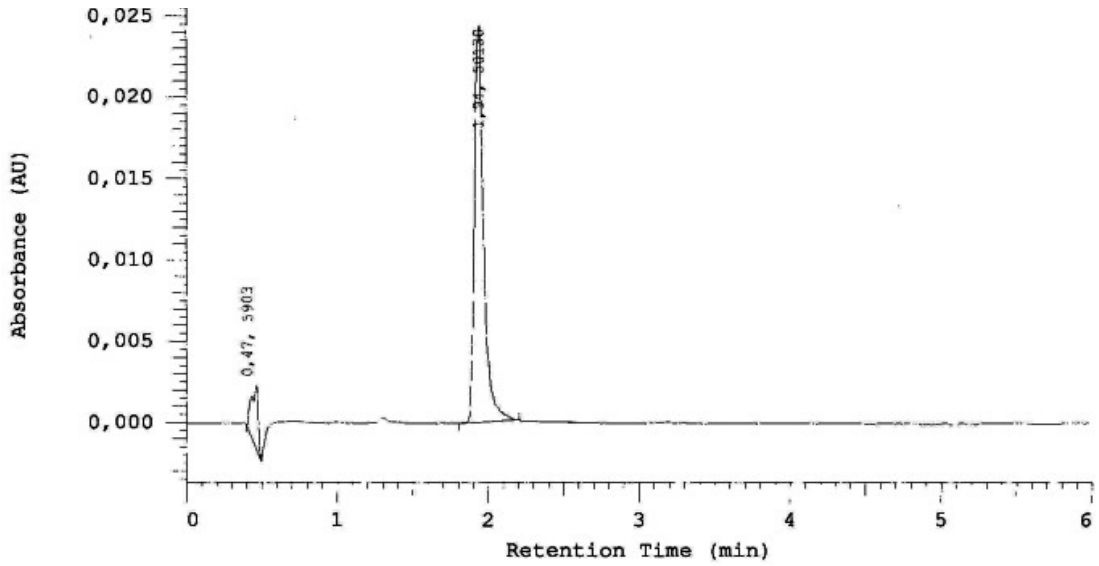


D: Rt = 1,17, Area = 158947

Figure 8. Representative chromatograms of flurbiprofen std (E), flurbiprofen sample (F) with Rt and area

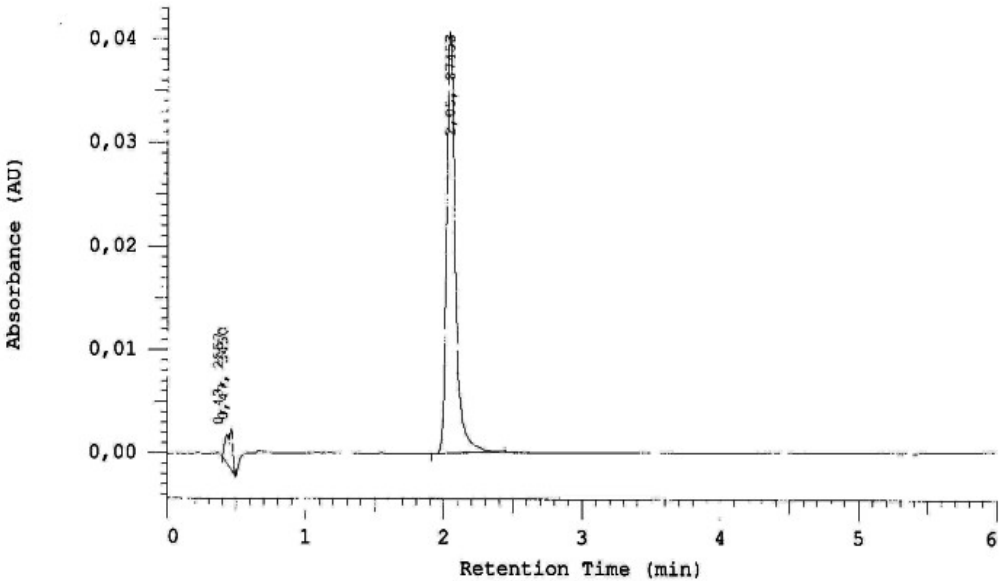


E: Rt = 1,94, Area = 48025

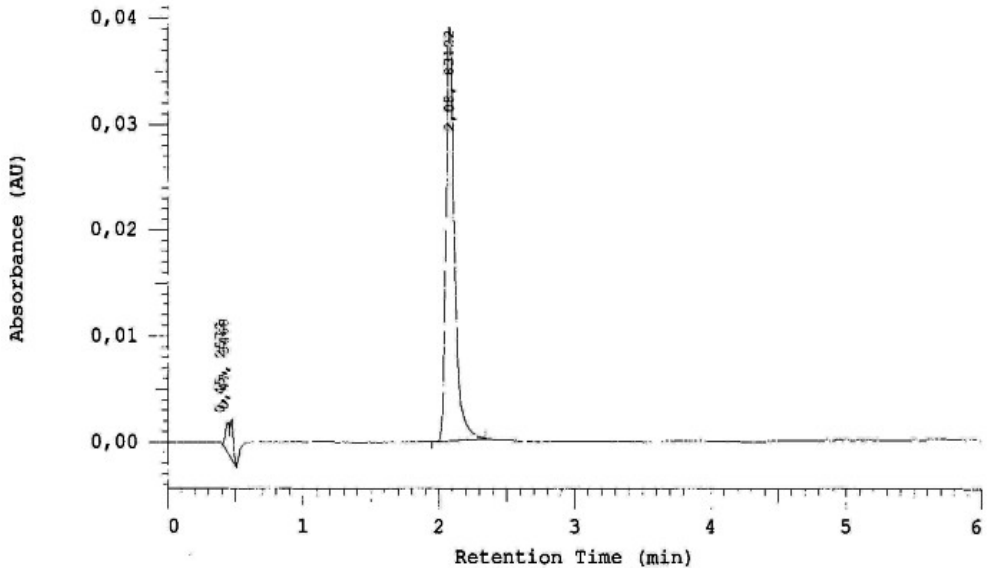


F: Rt = 1,94, Area = 50792

Figure 9. Representative chromatograms of flutamide std (G) and flutamide sample (H) with Rt and area

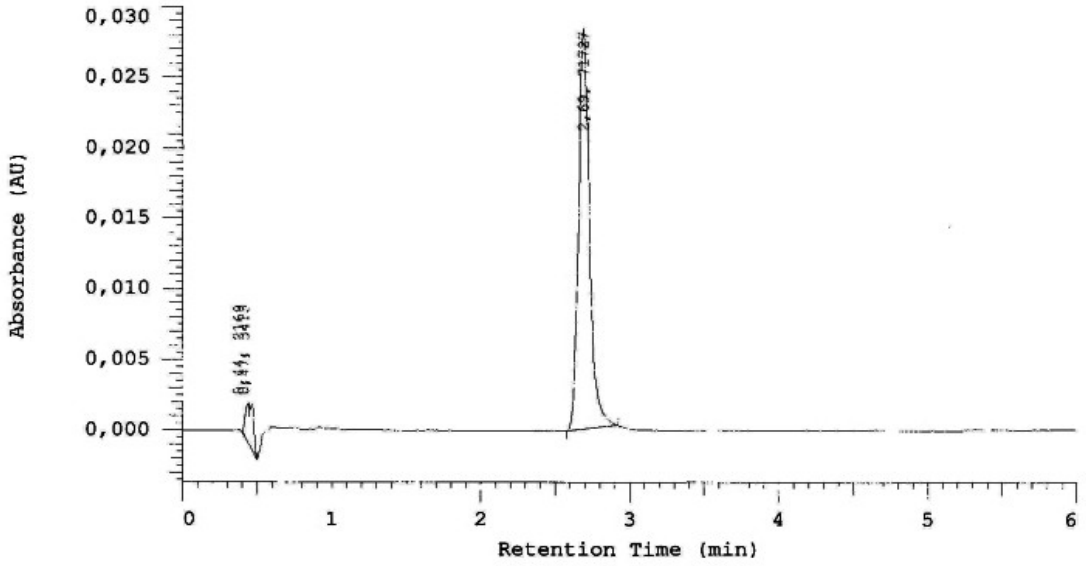


G: Rt = 2,07, Area = 86375

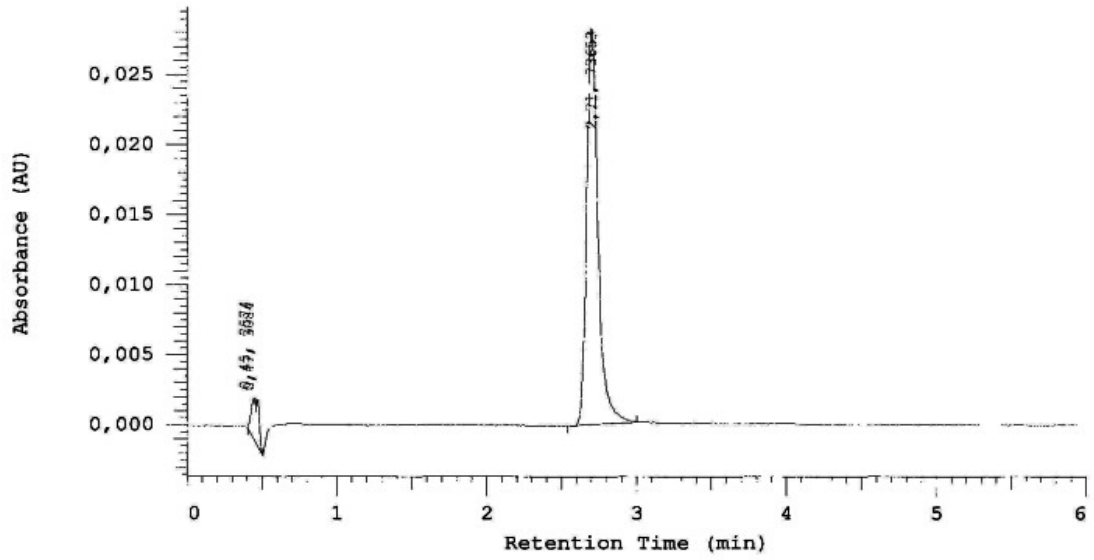


H: Rt = 2,07, Area = 82463

Figure 10. Representative chromatograms of ibuprofen std (I) and ibuprofen sample (J) with Rt and area

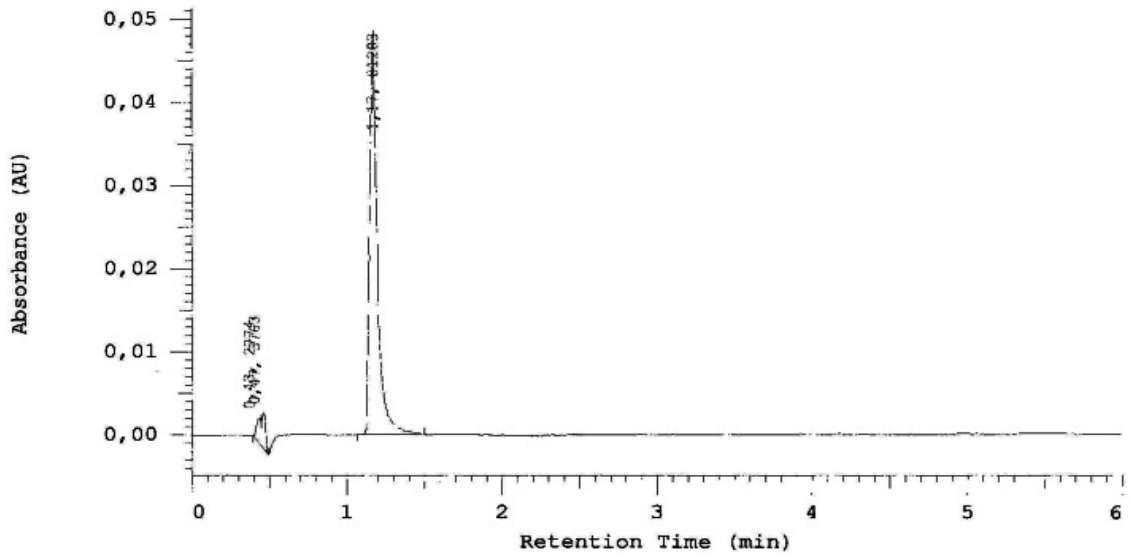


I: Rt = 2,68, Area = 72128

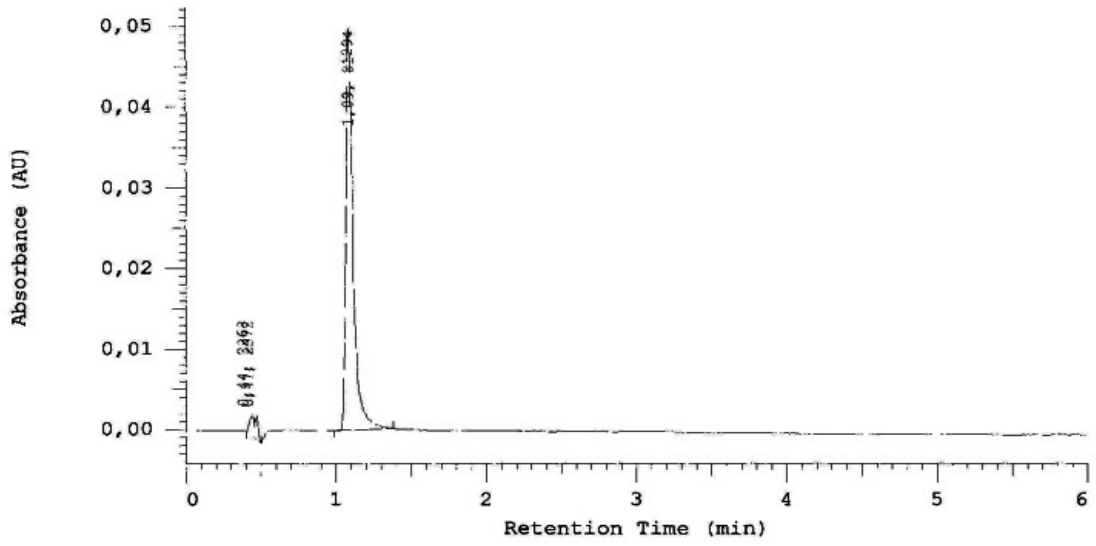


J: Rt = 2,70, Area = 73410

Figure 11. Representative chromatograms of ketoprofen std (K) and ketoprofen sample (L) with Rt and area

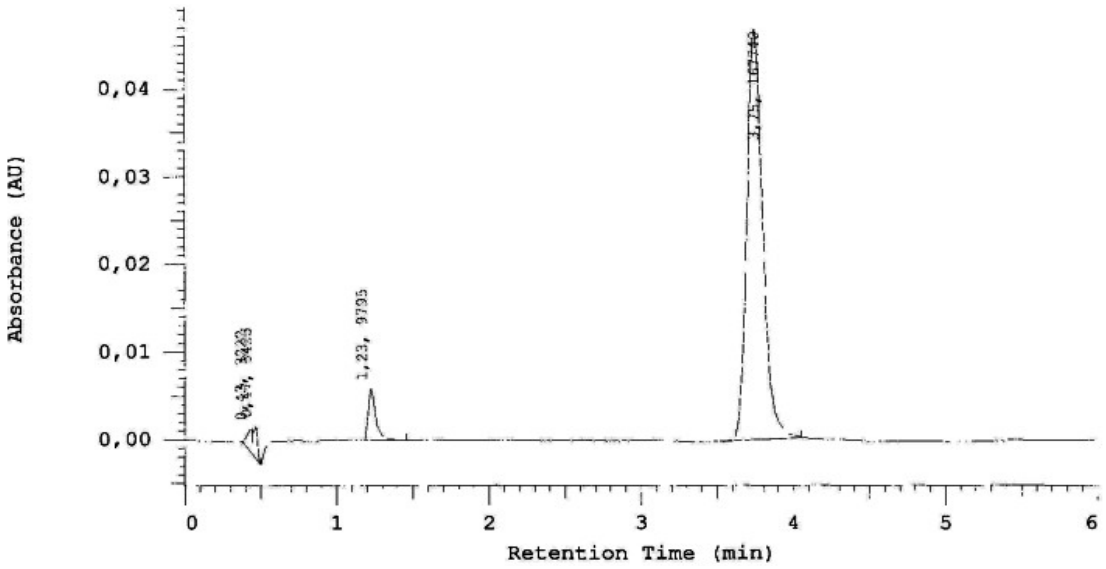


K: Rt = 1,17, Area = 80208

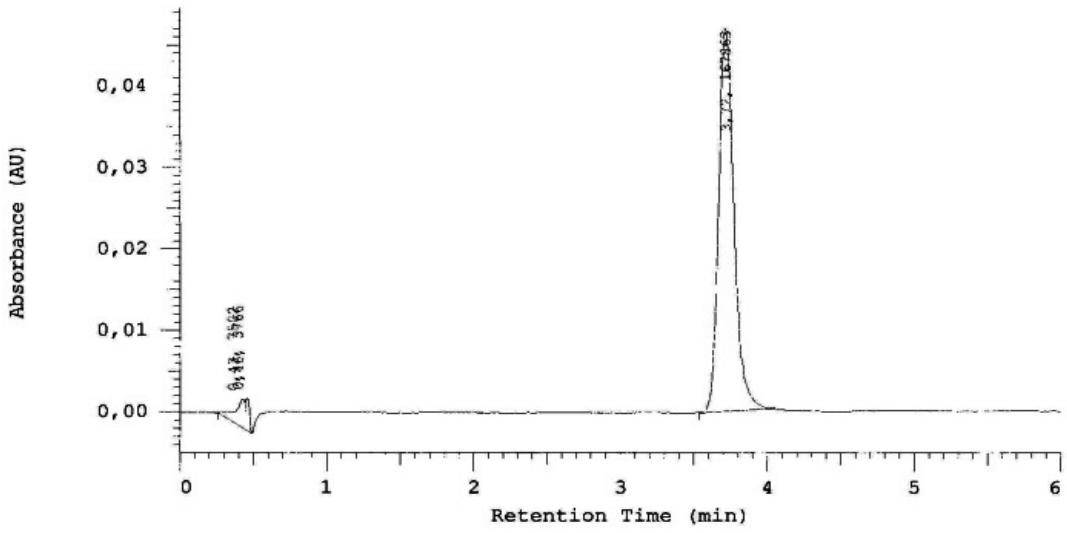


L: Rt = 1,13, Area = 81895

Figure 12. Representative chromatograms of mefenamic acid std (M) and mefenamic acid (N) with Rt and area

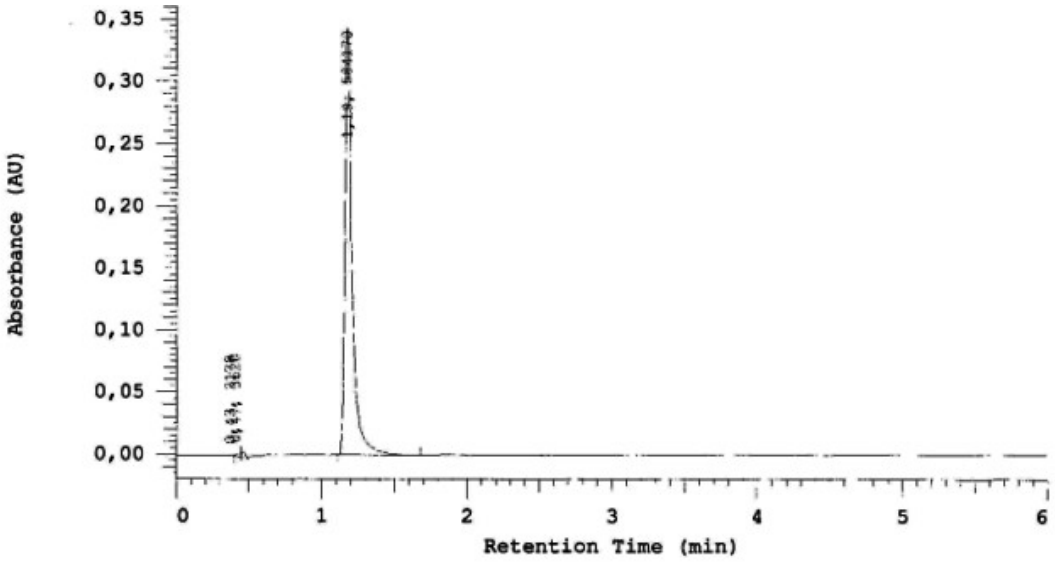


M: Rt = 3,71, Area = 168769

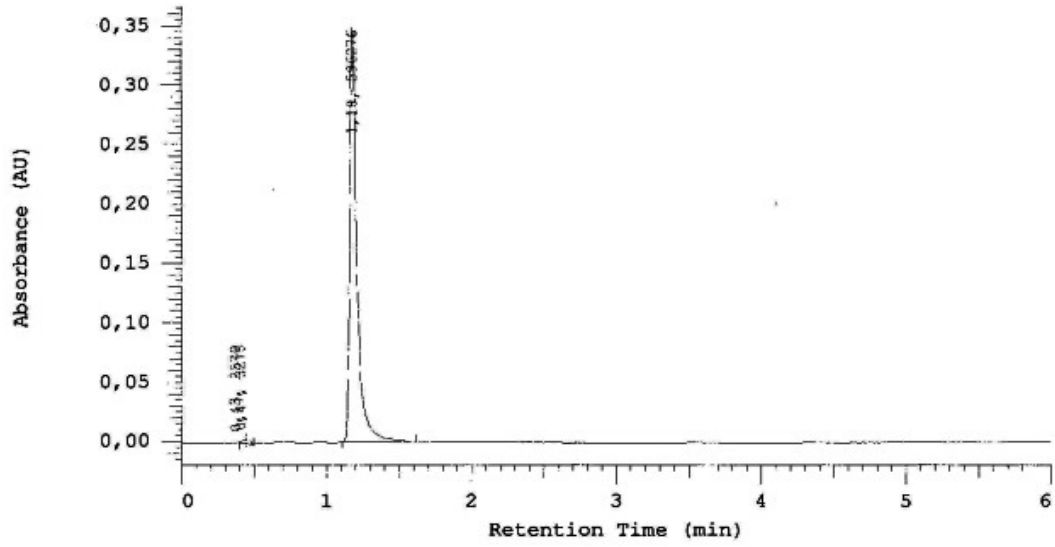


N: Rt = 3,71, Area = 166466

Figure 13. Representative chromatograms of naproxen std (O) and naproxen sample (P) with Rt and area

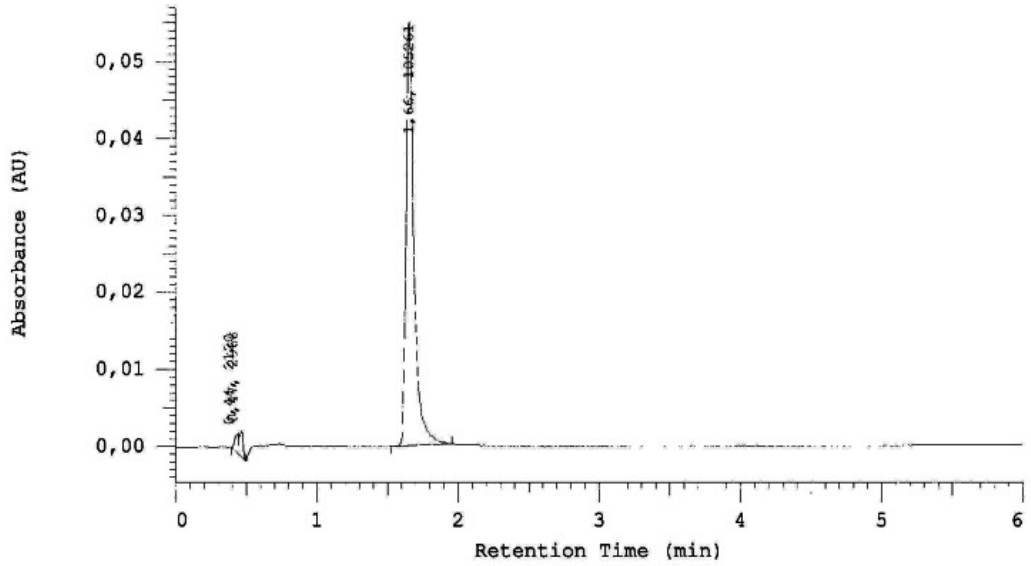


O: Rt = 1,18, Area = 169965

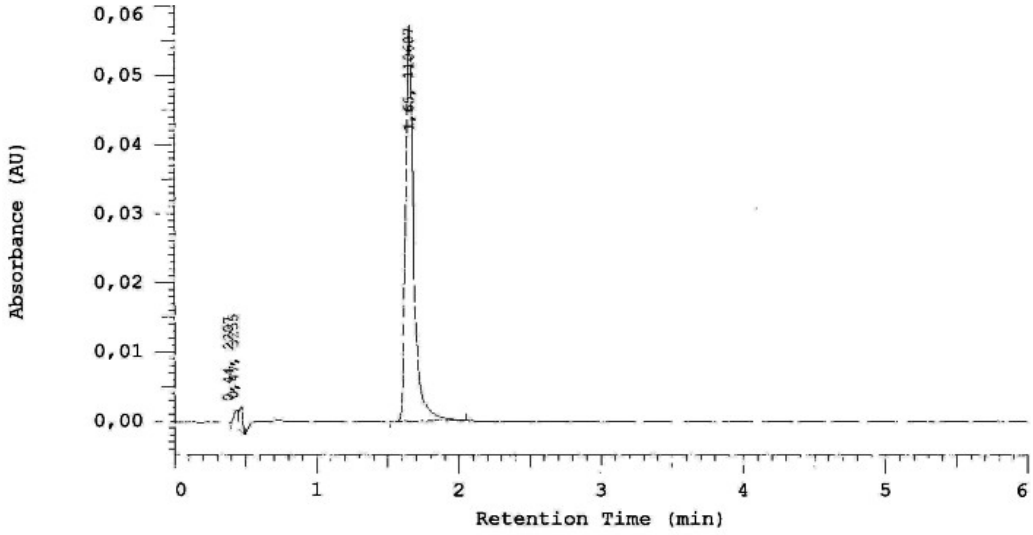


P: Rt = 1,18, Area = 594308

Figure 14. Representative chromatograms of nimesulide std (Q) and nimesulide sample (R) with Rt and area

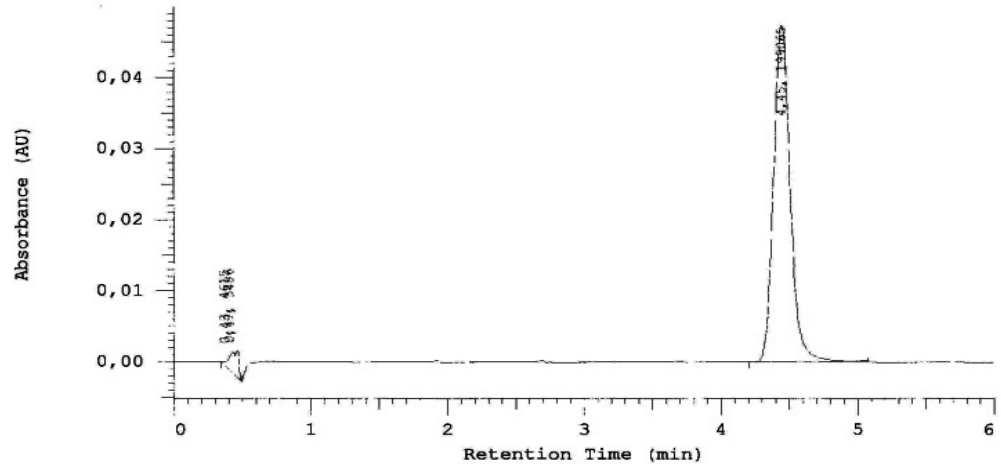


Q: Rt = 1,65, Area = 105481

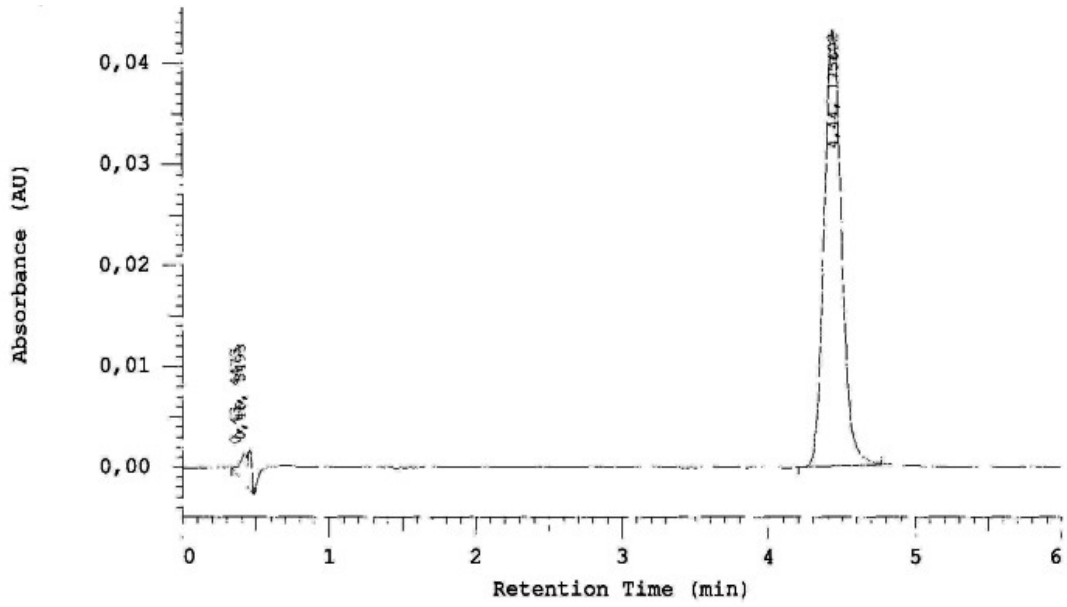


R: Rt = 1,65, Area = 109431

Figure 15. Representative chromatograms of tolfenamic acid std (S) and tolfenamic sample (T) with Rt and area



S: Rt = 4,45, Area = 199065



T: Rt = 4,47, Area = 179347

Previous results shows that all tested compounds are thermal stable.

4. Conclusion

The main aim of this work was to find, validate an useful HPLC method for group of compounds to measure their potential thermal instability.

This work is divided into three parts from this reason.
It the first part it was worked with wide group of 38 active substances. It was necessary to gain spectra and download them to the library of HPLC equipment for final comparing of stability. These compounds are listed in the chapter Chemicals, and also in one other chapter there are divided according to their therapeutic application. All compounds have variable pKa values, structures, molecular weight, and UV spectra. We measured Rt of all. Rt values depend on composition of mobile phase. Due to this we used 8 different mobile phases with variable ratios of ACN and the buffer. This is stated in Table 4. Rt values are in Table 5. Based on these results we decided to continue with the group of 10 active substances – diflunisal, fenbufen, flurbiprofen, flutamide, ibuprofen, ketoprofen, mefenamic acid, naproxen, nimesulide, and tolfenamic acid. The suitable ratio of ACN 90% and 50mM phosphate buffer pH 2,1 was 57: 43. All compounds except flutamide are NSAIDs with pKa around 4, flutamide has it much more higher around 13. We could not explain that.

In the second part we validated this chosen method. It was worked only with 10 selective compounds. Their characteristics are stated in chapter 2. 4. Results did not end well. During the validation process it was made one systematic mistake. It was used plastic pipettes all the time without any changes instead of glass pipettes. All process was done this way. It caused incorrect and inaccurate measurements. This mistake was proved by another experiment before next measurements. The results are stated in chapter Re-measuring. Another possible explanation for inaccuracy of several drugs can be in their labile characteristic in acidic environment. These problems were eliminated by using strictly glass pipettes during lab work and sample preparation right before use.

In the third part the comparing Rt and purity proved that at least in nine substances there is no degradation after heating the sample during an amorphous state preparation. There are no extra peaks, no different Rt and purity is still high. In two cases there are unexpected small peaks. There is the small peak in time 1,23 in mefenamic acid std but there is no peak

in mefenamic acid sample. This could be explaned by contamination of sample. Also the small peak in time 3,75 appeared in fenbufen sample. Together with lower stability and purity it could indicate small degradation. Next explanation is contamination. This peak appeared in more chromatograms than just in samples that went through heating.

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